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CHARACTERIZATION OF BOVINE BRAIN ATPASE

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The purified bovine brain ATPase has been shown to be a high-affinity, low capacity pump, and consists of two proteins. A labile, high affinity dihydropyridine receptor activity has been associated with this ATPase activity. This study has shown that the purified (Ca^{2+} + Mg^{2+})-dependent ATPase can be immobilized, and that changes can be detected on the surface as increases in phosphate released and/or capacitance differences after interaction of the complex with a test ligand.

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PREFACE

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on Revision of the Guide of Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

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CHARACTERIZATION OF BOVINE BRAIN ATPase

1. NATIVE SYNAPTIC MEMBRANES

The (Ca^{2+} + Mg^{2+})- dependent ATPase-Calcium Channel Complex is found at the synaptic termini of neurons(1). With the exception of Mojave toxin characterization, all work was carried out using bovine synaptic preparations due to the requirement for large quantities of tissue. Mojave toxin characterization used rat brain as a source of protein because a dihydropyridine binding assay had previously been described with rat brain tissue(2). Methodologies employed for preparation of membranes as well as those used for measurement of ATP hydrolysis and dihydropyridine binding are shown in the Appendix.

1.1 PARTIAL CHARACTERIZATION

Initially, catalytic properties of bovine brain, synaptic membrane (Ca^{2+} + Mg^{2+})-dependent ATPase preparations were established. As shown in Figure 1, two regions of saturation were observed with regard to release of phosphate from ATP when assayed at pH 7.2 in the presence of 2.52 μ M Ca^{2+} and 200 μ M Mg^{2+}. One, a high affinity activity, saturates at very low ATP concentration (approximately 15 μ M). A second but lower affinity (Ca^{2+} + Mg^{2+})-dependent ATPase activity was observed to saturate at higher ATP concentration (approximately 40-45 μ M).

Characterization of these activities with regard to their dependence upon pH is shown in Figure 2. Two distinct profiles were observed depending upon ATP concentration. When assayed in the presence of 200 μ M ATP (open circles), hydrolysis of ATP was observed to occur over a broad range of pH with maximum hydrolysis occuring at pH 7.0. Assays

conducted in the presence of low ATP concentration, such as 12.5 μM (closed circles), also indicated a broad pH dependence with one maxima at 7.4 and a second at 7.8. Assay of synaptic membrane homogenate preparations at pH 7.0 in the presence of 25-125 μM ATP and graphical analysis of the ratio of velocity and substrate concentration versus that of velocity, indicated K_m and V_{max} values of 24 μM and 110 nmoles/min/mg protein, respectively (Figure 3). Synaptic membrane homogenates assayed at pH 7.4 in the presence of low but increasing concentrations of ATP (2.5 - 15 μM in Figure 4), indicated saturation to occur at approximately 7.5 μM. As shown in Figure 4, Eadie-Hofstee graphical analysis of these data (insert), indicated K_m and V_{max} values of 3.2 μM and 59 nmoles/min/mg protein, respectively.

Evaluation of the dependence of hydrolysis of ATP on free calcium at low (12.5 μM) and high (200 μM) ATP concentrations buffered at optimal pH is shown in Figure 5A. The high affinity activity (closed circles) appeared to be inhibited by increasing Ca^{2+} concentrations in contrast to the low affinity activity (open circles). Shown in Figure 5B is an Eadie-Hofstee graphical analysis of kinetic data. Both catalytic activities exhibited a K_m value of 0.24 μM for calcium; however, V_{max} values obtained using low and high ATP concentrations differed significantly (128 and 49 nmoles/min/mg protein, respectively). Hill Plot analysis (Figure 5C) indicated slopes of 1.1 and 0.5 under low and high affinity assay conditions, respectively.

The role of Mg^{2+} in the phosphorylation-dephosphorylation ATPase reaction sequence is very complex(3). Under physiological conditions, Mg^{2+} is part of the substrate, Mg-ATP. Shown in Figures 6 and 7 is the dependence of hydrolysis of ATP upon magnesium. Eadie-Hofstee graphical

analysis at low and high ATP concentration (insert) revealed K_m values of 112 and 18 μM, respectively. V_{max} values were similar to those previously derived from Ca^{2+} and ATP saturation plots. Summarized in Table I are the kinetic constants obtained from assays using optimal hydrogen ion, Ca^{2+} free, Mg^{2+} free and ATP concentrations. These conditions are referred to as low and high affinity assay conditions.

In order to ascertain if these activities were mitochondrial in nature, both low and high affinity ATPase assays were performed in the presence of several mitochondrial inhibitors at concentrations sufficient to block Ca^{2+} uptake by brain mitochondria(4). As indicated in Table II, KCN, NaN_3 and ruthenium red did not inhibit any of these activities. Rotenone and oligomycin in general inhibited the activity 10-20%.

In an attempt to determine if one or both of these activities were calmodulin-stimulated(5), synaptic membrane homogenates were assayed under low and high affinity conditions in the presence of increasing amounts [μM] of trifluoperazine. Trifluoroperazine has been claimed to block various Ca^{2+} dependent processes by its direct interaction with calmodulin(6). As indicated in Figure 8, both activities were inhibited maximally (approximately 50%) at 30 μM trifluoroperazine. Treatment of synaptic membranes with EGTA for removal of endogenous calmodulin(7) may not have removed all of the tightly bound calmodulin which may be inaccessible to added trifluoperazine(8), thus reducing the degree of inhibition.

The low affinity activity was observed to be very sensitive to vanadate (see Figure 9A), revealing its plasma membrane in origin(9). The high affinity activity required approximately twice as much inhibitor to reduce the activity to 50% that of control. In the presence of 10 μM

vanadate both activities were reduced 90% or more. Lanthanum (La^{3+}) is another inhibitor with considerable specificity for (Ca^{2+} + Mg^{2+})-dependent enzymes(10). Lanthanum reduced both activities similarly but was not as effective at lower concentrations (see Figure 9B).

Lotersatajn and Pecker(11) observed various cations to uncouple Ca^{2+} stimulated (Ca^{2+} + Mg^{2+})-ATPase in rat liver plasma membranes. An extensive list of various cations (mono-, di- and trivalent) and their effects upon ATP hydrolysis are shown in Table III. The free cationic concentration is considerably higher than those free cation concentrations used in the Lotersztajn and Pecker study. Perhaps those cations, such as those preferentially inhibiting the high affinity activity which is inhibited by free Ca^{2+} in excess of 2.52 μM, have not effectively increased the free Ca^{2+} concentration by combining with EGTA. Both low and high affinity activities were inhibited by exogenous cations to various degrees; however, sodium and ammonium ions preferentially inhibited the low affinity activity more than 90% while leaving greater than 90% of the high affinity activity intact. Addition of cesium, aluminum, and manganese resulted in almost total inhibition of the high affinity activity. Manganese and aluminum ions reduced the low affinity activity approximately 20%. Only barium treatment resulted in stimulation.

A thermal lability study revealed the two activities to exhibit differences with regard to thermal denaturation (Figure 10). The low affinity activity (open circles) gradually lost activity when incubated at 45°C for increasing periods of time. In contrast, the high affinity activity (closed circles) was not affected until 2.5 minutes, after which approximately 50% of the activity was lost.

Binding studies with radioactive derivatives have shown that the dihydropyridine derivatives recognize a binding site distinct from that of verapamil and diltiazem(12). Verapamil and diltiazem respectively inhibit and stimulate nitrendipine binding allosterically, suggesting actions at different sites(13). The effects of the allosteric calcium channel modulators, verapamil and diltiazem, on these ATPase activities are as follows. As shown in Figure 11A, diltiazem stimulated the high affinity ATPase activity approximately 40%, while stimulating the low affinity activity approximately 10%. Stimulation of the low affinity activity was followed by a gradual but significant decline in ATP hydrolysis. Verapamil inhibited only the low affinity activity with no apparent effect upon the high affinity ATPase activity (Figure 11B). It is possible that the calcium channel allosteric site for diltiazem and the high affinity ATP catalytic conformation(s) or site may be located very close to one another or possibly the conformation(s) arising from direct interaction with diltiazem may be more optimal for ATP hydrolysis.

1.2 MOJAVE RATTLESNAKE TOXIN STUDIES

We carried out a series of experiments examining the effects of Mojave toxin, a potent neurotoxin on both channel binding and ATP hydrolysis. Mojave toxin was isolated and purified from Crotalus scutalatus scutalatus venom by immunoaffinity chromatography(14). These studies were all carried out using synaptic membranes from rat brain as the source of protein. As shown in Figure 12, purified snake toxin stimulated the (Ca^{2+} + Mg^{2+})-dependent ATPase approximately 2-fold (open circles) with no effect upon the Ca^{2+} independent, Mg^{2+}-dependent activity (closed circles).

Examination of the effects of increasing amounts of purified Mojave toxin on binding of the calcium channel blocker, nitrendipine is shown in Figure 13. Addition of 10μ g of toxin resulted in greater than 90% inhibition of ligand (nitrendipine) binding. Addition of 20μ g resulted in complete inhibition of binding. Summarized in Table IV are binding data obtained in the presence and absence of unlabelled ligand. Synaptic membranes were observed to bind specifically 190.4 fmoles/mg protein (Experiments A and B). In the presence of unlabelled nitrendipine, 7.6 fmoles (^{3}H)-nitrendipine per mg protein, approximately 3.8% total bound ligand, were observed to bind non-specifically. As shown in experiment C, purified Mojave toxin behaved in an identical fashion as unlabelled ligand, giving rise to 7.5 fmoles (^{3}H)-nitrendipine bound per mg protein. The ligand bound in the presence of toxin appears to be nonspecifically associated as evidenced by the results of Experiment D. Only very small amounts of (^{3}H)-ligand (0.8 fmole/mg protein) were observed to bind to the toxin as indicated by Experiments E and F.

Since Mojave toxin is associated with a phospholipase A_2 activity(15), we investigated the possibility of using 4-bromophenacylbromide, a known inhibitor of phospholipase A_2 activity(16) to discern the possible effects of the purified toxin on synaptic membranes. As shown in Table V, at low concentrations (1μ M), the inhibitor had little effect on either (Ca^{2+} + Mg^{2+})- dependent ATPase activity or nitrendipine binding. In contrast, 100μ M bromophenacylbromide resulted in significant inhibition of both parameters. Since both ATPase activity and nitrendipine binding were inhibited significantly by bromophenacylbromide at a concentration (100μ M) previously shown to be effective in inhibiting 25-50 μg purified cobra venom phospholipase(16), the use of this inhibitor in

finding the possible role of endogenous phospholipase activity was greatly diminished. Additional experiments indicated the toxin to be specific for the Ca^{2+} channel(17).

The effects of two calcium antagonists that allosterically affect nitrendipine binding(13) are shown in Figure 14. Purified snake toxin (10 µg) resulted in a 90% increase of specific (^3H)-ligand binding (Control plus Toxin; closed circle). Verapamil, in the absence of toxin, characteristically reduced binding by approximately 30% (open squares). In the presence of both toxin and verapamil, nitrendipine binding was reduced greater than 90% (Verapamil plus Toxin; closed square, Figure 14). Diltiazem, a positive allosteric modulator, characteristically increased nitrendipine binding approximately 40% as compared to control (open squares). However, in the presence of toxin and diltiazem, nitrendipine binding was reduced approximately 90% (Diltiazem plus Toxin, closed triangles).

2. SOLUBILIZATION AND PURIFICATION

All studies described thus far utilized membrane bound protein. In order to accurately define the biosensing elements in chemical terms, removal from native membrane as well as purification of the protein complex was required. Therefore, a series of experiments was carried out in order to determine characteristics of detergent solubilized (Ca^{2+} + Mg^{2+})-dependent ATPase. These studies facilitated the required transition from experiments utilizing impure, membrane bound protein to those employing detergent solubilized, affinity purified preparations.

2.1 COLUMN PURIFICATION

Much effort was focused on Calmodulin-Sepharose affinity column purification of the (Ca^{2+} + Mg^{2+})-dependent ATPase. In our studies, we have utilized the method of Niggli, et al (18) with only slight modifications developed in this laboratory. All of the purification procedures developed in this laboratory are detailed in the Appendix.

Examination of Calmodulin-Sepharose affinity column purified protein by SDS-Polyacrylamide gel electrophoresis revealed two silver staining bands (19,20); one band of 128,600 molecular weight and a second of 98,000 molecular weight (gel scanned using a Hoefer GS-300 Densitometer and GS-350 Data system). Integration of respective areas on the gel, indicates the 128,600 dalton molecular weight species to constitute approximately 65% of the total protein applied to the gel; whereas, the 98,000 dalton band constitutes approximately 35% of the total protein applied. To date, we have not been able to incorporate ^{32}Phosphorous into alkali labile, acylphosphate enzyme intermediates (21), thus facilitating the identification of one or both of these protein staining bands as having ATPase activity. This is discussed later with regard to kinetic observations (see 2.4)

We observed elution of both nitrendipine binding and ATP hydrolysis following addition of EDTA to the column equilibration buffer (Figure 15). When Ca^{2+} or EDTA concentrations were varied, ATP hydrolysis (open triangles) and nitrendipine binding (open circles) elution profiles differed (Figure 15). All elution profiles indicated ATP hydrolysis to coelute with the major protein peak (closed circles) in contrast to nitrendipine binding which was consistently observed in fractions with little protein. Nitrendipine binding activity was observed to be very

sensitive to detergent concentration. For example, treatment of synaptic membrane preparations with detergent (Triton X-100) at one concentration (0.15% v/v) preserved ATPase activity, while inhibiting dihydropyridine binding.

A complicating feature encountered with calcium channel ligand binding activity was the extreme lability of the high affinity dihydropyridine receptor following solubilization. Binding is lost within 60-120 minutes of elution. Minor changes in detergent, phospholipid, and cation concentrations resulted in complete inactivation of the receptor.

Detergent solubilization and affinity column purification also presented lability problems with regard to ATP hydrolysis, but much less so than with the high affinity dihydropyridine receptor. Optimal conditions giving rise to maximum ATP hydrolysis were evaluated. Initially, cation concentrations in eluted fractions exhibiting ATP hydrolysis could at best only be approximated. This was due to the fact that actual concentrations of EDTA, Ca^{2+}, and Mg^{2+} in eluted column fractions were uncertain. Considerable variation occurred depending upon relative protein concentration per fraction. This problem necessitated removal of cations or chelators by dialysis. At minimum, an approximation of optimal cation/chelator concentration was required in order to effectively immobilize the purified protein complex.

2.2 DIALYSIS

In order to remove Ca^{2+}, Mg^{2+} and the chelators EDTA or EGTA introduced during affinity column purification, dialysis was carried out using a low molecular weight (12,000 dalton molecular weight cutoff) graded porosity membrane and Protocon Dialysis apparatus. In developing this

procedure, it was observed that sucrose (0.6 M) stabilized the enzyme complex (Figure 16 open circles). Column fractions maintained at 5°C in the presence of 0.01 M Tris buffer, pH 8.2 (containing asolectin, dithiothreitol, and Triton X-100) but no sucrose (closed circles), resulted in lower activity. Shown in Figure 17 is the effect of increasing storage time at 5°C following dialysis. These data indicate that the enzyme is fully active 4-6 hours following dialysis. Dialysis resulted in achieving significant reduction of ions (Mg^{2+}) and chelators as determined by Atomic Absorption (Table VI). Surprisingly, the amount of Ca^{2+} before and after dialysis was almost identical. This indicates that much of the Ca^{2+} is bound to the purified protein. Ca^{2+} concentrations following dialysis is approximately 175 μM. However, when diluted under optimal assay conditions (1:40, 50 μ gms protein) and accounting for the presence of EDTA, the amount of free Ca^{2+} is 3.4 nM. This is significantly lower than the amount of Ca^{2+} observed to be present following washing of membrane preparation(10). Additionally, this indicates that our buffering conditions are ideal since we obtain a $K_{Ca^{2+}}$ value of 24 nM.

Free cation concentration is very dependent upon pH, ionic strength, and chelator concentration. Therefore, it is necessary to account for all these parameters to assess free cation concentration. Previously, we had calculated free cation concentration by the method of Bartfai(22). Removal of ions and chelators from the purified preparations now makes it possible to determine accurately free cation concentrations required for maximum enzymatic activity. Using the method of Fabiato and Fabiato(23), dissociation constants for chelator-ion complexes are adjusted for pH and ionic strength. Thus, cation and chelator concentrations required for a desired free ion concentration can be accurately calculated. This method of

calculation can resolve as many as three ionic species in the presence of as many as four different chelating reagents. Using this method of calculation, adjustments were made in order to correct kinetic data derived from earlier experiments.

2.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In order to purify protein more rapidly as well as to develop an alternative method for removal of ions and chelators introduced during the affinity chromatographic procedure, column fractions exhibiting ATP hydrolysis were subjected to High Performance Liquid Chromatography (HPLC). Initially, HPLC of protein preparations was carried out using an ion exchange column (Waters, DEAE 5PW) previously equilibrated in buffer with elution of protein by pH and salt concentration gradients both singly and in combination. High UV absorbance resulting from the required inclusion of detergent (Triton X-100) in the buffer prevented monitoring of eluted protein at 280 nM. Triton X-100, even when saturated (completely reduced, Sigma Chemical Company, St. Louis, Missouri) made detection of protein at 280 nM practically impossible. However, enzyme (Ca^{2+} + Mg^{2+})-dependent ATPase activity could be effectively monitored spectrophotometrically (Figure 18). As indicated in the figure, a minimum of three activity peaks were observed to elute from the column. Because of elution problems, the respective peaks were not pooled and examined further.

Although these experiments were partially successful, we concluded that ion exchange chromatography was inappropriate due to poor resolution and significant loss in specific activity (nmoles phosphate released/min/mg protein). Decreased resolution is probably attributable to interactions of detergent and phospholipids which are required for

stabilizing the solubilized protein. Furthermore, decreased ATP hydrolysis may also arise from protein-column interactions giving rise to conformational changes.

In a further attempt to utilize the rapid nature of HPLC, gel filtration was pursued. Sucrose was included in equilibrating and eluting buffers to help stabilize activity during purification. Again, poor resolution was observed with much of the activity passing through the columns (Waters, 300 SW) in the void volume (Figure 19, designated v_o). Because this particular gel filtration column specifically resolves proteins varying in molecular weight up to 300,000 daltons, it appears that the combination of detergent, phospholipid, and protein results in formation of micelles in excess of 300,000 daltons as evidenced by detection of activity in the calculated column void volume.

These experiments indicate that HPLC used under the described conditions, although faster than Calmodulin Affinity Column Chromatography, does not yield high specific activity protein. Many of the recovery related problems encountered with the very labile dihydropyridine receptor may be resolved by more rapid means of purification. The use of HPLC has much potential; however, optimal conditions of detergent extraction and purification must be established.

2.4 PURIFIED ENZYME KINETICS

A complete kinetic study was made in order to optimize assay conditions in preparation for plate-immobilization studies. These data are critical in defining the biosensor, as they provide an accurate means of following protein attachment onto derivatized surfaces. The dependence of catalytic activity on the concentrations of hydrogen ions, ATP, Ca^{2+} free

and Mg^{2+} free were determined as shown in Figures 20-23. ATP hydrolysis was observed to occur over a broad pH range (6.8-7.6, Figure 20). As previously indicated, chelation of cations is a pH dependent process. Thus, at each pH tested, Ca^{2+} and Mg^{2+} concentrations were adjusted such that the amount of respective free cations was kept constant at all hydrogen ion concentrations tested.

The dependence of hydrolysis on ATP concentration is shown in Figure 21. The K_m for ATP (157 μM) is rather high and may present problems with regard to phosphorylation of intermediates. Typical phosphorylation studies are carried out using ^{32}-P (ATP) of approximately 5-7 μCi/nmol (specific activity) (18). Thus, the amount of unlabelled ATP that must be added to bring the concentration of ATP is approximately that observed for the K_m results in substantial dilution of isotope (approximately 0.05 μCi/nmol, specific activity).

The dependence of ATP hydrolysis upon free Mg^{2+} and Ca^{2+} is shown in Figures 22 and 23, respectively. Eadie-Hofstee graphical analysis (inset, Figures 21-23) indicated no obvious deviation from linearity. Ikemota and coworkers(24) have observed positive cooperativity (Hill coefficient of approximately 2.0). There are no breaks in the Eadie-Hofstee plot for Ca^{2+}. ADP reduces ATP hydrolysis more than 70%, suggesting that the purified preparation functions in forward and backward directions; this is attributable to a preponderance of $2Ca^{2+}$. E-P conformer at equilibrium in the formalism of the reaction scheme involved two configurations defined as E_1 and E_2(25). A summary of derived kinetic constants (K_m) is presented in Table VII.

Alterations in nucleoside triphosphate substrate specificity arising from detergent solubilization of protein was also examined (Table

VIII). As indicated in the table, purified protein exhibited no specificity with regard to pyrimidine nucleoside triphosphates. Only residual hydrolysis was observed for the other purine nucleoside triphosphates inosine and guanosine.

Sensitivity to various inhibitors is shown in Figure 24. The purified preparation remains sensitive to vanadate although not as sensitive as the protein, when associated with the membrane. Since the binding sited for inorganic phosphate is likely to be in the vicinity of the phosphate acceptor aspartyl group, it appears that detergent extraction results in alterations of the vanadate binding site thus increasing the amount required for 50% inhibition. We examined the effects of various phospholipids on catalytic activity (Table IX). With the exception of phosphatidic acid, all diacyl phospholipids tested were observed to be stimulatory. Lysophospholipids exhibited varied effects. Although the differences are small (12, 13, and 2% respectively), fatty acid chain length is important as evidenced by the inhibitory effects of the shorter myristoyl (C = 14) and lauroyl (C = 12) compounds as compared to the longer species, oleoyl (C = 18). This suggests that the presence of the double bond does not interfere in the inhibitory process. The oleoyl derivative of lysophosphatidic acid exhibited no inhibition of ATP hydrolysis in contrast to the completely acylated derivative of phosphatidic acid. Apparently the removal of the acyl moiety in the sn-2 position of the glycerol phosphate backbone of phosphatidic acid is important for inhibition.

In summary, chain length as well as degree of saturation are probable determining factors rather than solely degree of acylation. It is interesting to note that all lysophosphatidylcholine compounds tested were inhibitory in contrast to phosphatidylcholine which was clearly

stimulatory. Lysophosphatidylethanolamine is less stimulatory than phosphatidylethanolamine, as was lysophosphatidlyserine when compared to phosphatidylserine. Incorporation of the purified protein complex into artificial membrane bilayers or phospholipid vesicles could provide a mechanism for further stabilization of biosensor elements.

The presence of covalently linked carbohydrate to the purified (Ca^{2+} + Mg^{2+})- dependent ATPase was evaluated by passage of purified protein through immobilized lectin affinity chromatography columns. As indicated in Table X, essentially quantitative recovery of activity was obtained following washing and elution of the protein off of the various lectin columns. These findings indicate carbohydrate microheterogeneity with regard to covalently linked oligosaccharide structure. Although Concanavalin-A has an affinity for the trimannosyl-core of N-type glycosides(26), the low percent binding cannot be interpreted to indicate low amounts of typical N-type glycoside because chromatography was carried out in the absence of Ca^{2+} and Mn^{2+}. These two cations are required for maximal Concanavalin-A binding of glycoconjugates(27). This was done because of the chelation effects of added cations in the reaction mixtures. The very low cationic concentration
may be suggestive of additional hydrophobic interactions which may be partially responsible for binding of protein to Concanavalin-A(28).

In summary, the purified preparation has a carbohydrate profile consistent with a complex structure (presence of galactose, mannose and sialic acid). This structure raises the possibility of immobilizing via immobilized lectins. Since we have not carried out a complete carbohydrate analysis of the purified protein, we cannot rule out the presence of O-type

oligosaccharides in view of the fact that the protein binds to Soy Bean Agglutinin (SBA).

2.5 CHEMICAL DERIVATION

In order to attach protein to chemically derivitized silicon oxide surfaces, it was necessary to determine the effects of succinic anhydride and carbodiimide on ATP hydrolysis. This was required due to the diverse chemical effects these reactive reagents have on amino acid side chains which alter protein function (i.e., binding of cofactors, substrates, and ligands).

2.5.1 The Effects of Succinic Anhydride and Carbodiimide

Initial difficulties with reagent solubility were resolved by dissolving succinic anhydride in 10% (v/v) DMSO. Using native membrane preparations, high concentrations of these reagents (succinic anhydride and carbodiimide) affected ATP hydrolysis (Figures 25 and 26 respectively) while nitrendipine binding remained unchanged (data not shown). An estimate of the stoichiometry of succinylation of detergent solubilized protein was obtained using ^{14}C-labelled succinic anhydride. We estimated incorporation of about 0.3% of the total label into protein. We purified the radioactively labelled protein by calmodulin affinity chromatography. Very little radioactivity was obtained in the column eluate, thus this particular approach was not pursued. In contrast to membrane bound protein which was inhibited by increasing amounts of succinic anhydride to stimulate ATPase activity slightly (Figure 27).

One key finding in this series of experiments was that asolectin provided a measure of protection to the protein during chemical treatment.

Furthermore, asolectin afforded restoration of enzymatic activity (open circles) following removal of much of the calmodulin, compared to activity of intact, synaptic membrane preparations (Figure 27, single point determination, closed circle). The difference between Figure 27 and 28 is the respective presence or absence of calmodulin before solubilization.

Many of the difficulties associated with enzyme sensitivity attributed to succinic anhydride derivatization were alleviated by inclusion of asolectin. Thus, we elected to bind the solubilized (pre- and post purified) protein via calmodulin previously immobilized on 3-aminopropyltriethoxysilane (APTES) derivatized plates. All subsequent procedures developed for the linkage of protein to APTES derivatized silicon oxide surfaces are described in the Appendix.

2.5.2 Immobilized Enzyme-Early Studies

Various techniques of immobilizing protein preparations on APTES derivatized surfaces have been investigated. Our initial approach was to link native membrane bound protein directly. A series of experiments using either calmodulin deficient(7) synaptic plasma membranes or detergent solubilized membranes was conducted. The immobilized protein was monitored by ATP hydrolysis. As indicated in Figure 28, the amount of enzyme immobilized was very small; specific activity was in nmoles/hr in contrast to the usual specific activity of nmoles/min. Due to the very small amount of protein immobilized, protein could not be quantitated. Furthermore, since the protein immobilized was a mixture and we do not have a mass measurement (μg, protein), it is not possible to derive any further useful stoichiometric data.

The hydrolysis of ATP by synaptic preparations is calmodulin stimulated(5). Furthermore, the protein is purified using immobilized calmodulin(18). Therefore, we were interested in using calmodulin coated biosensor surfaces in an affinity chromatography fashion. Linkage could occur via either electostatic interactions (interaction of the highly electronegative calmodulin molecule with the positively charged APTES coated surfaces) or covalent linkage of succinylated calmodulin by derivatization in the presence of water soluble carbodiimide. Shown in Figure 29 is the effect of increasing amounts of immobilized calmodulin on the adherence of solubilized protein as monitored by hydrolysis of ATP.

2.5.3 Capacitance Bridge-Biosensor Testing

Using the aforementioned methodologies, we conducted an experiment using a capacitance bridge and wired, biosensor plates supplied by Mr. Joe Wall of the Johns Hopkins Applied Physics Laboratory. Solubilization of affinity column purified enzyme and succinylation of calmodulin was carried out as described in Appendix. One hundred microliter aliquots containing 0.5 μg of succinylated calmodulin were tranferred onto each of four biosensor plates previously derivatized with APTES followed by addition of 10 μl of 10 mM carbodiimide. A parallel experiment monitoring ATP hydrolysis on APTES derivatized a SiO-APTES coated (0.5 x 0.5 inch) glass plate was also carried out. All plates were agitated for 40 minutes at room temperature, followed by washing (2x) with 0.01 M Tris-glycerol, pH 8.2 buffer.

Electrode bearing plates were overlaid with calmodulin affinity purified enzyme as follows: 2.5 ml aliquots from combined column fractions exhibiting high ATPase activity were added to tubes containing 12.5 mls of

0.01 M Tris-glycerol, pH 8.2 buffer with 0.015% Triton X-100 and 50 µl of 0.2 M $CaCl_2$. Plates were immersed in the protein solution and agitated for 10 minutes. Following washing, capacitance changes in air and in a fixed volume of 0.01 M Tris-glycerol, pH 8.2 buffer were monitored using a capacitance bridge as schematically described in Figure 30. A summary of treatment conditions of the plates is given in Table XI.

Following treatment for 15 minutes, plates were washed and capacitance monitored. As the data in Table XII indicate, capacitance decreased except in the presence of the purified snake toxin where an increase of approximately 1 nf was observed. This suggests that following administration of toxin, a possible loss of the protein from the derivatized surface occurred. This is consistent with the displacement of prtein (low dielectric constant) with water (high dielectric constant).

3. SUMMARY AND PROPOSALS

We have attempted to address several problems involving 1) receptor-protein characterization before and after detergent solubilization, 2) establishment of immobilization protocols, 3) partial characterization of a potential threat agent (Mojave Rattlesnake Toxin), 4) evaluation of a more rapid means of purification of receptor-protein, and 5) capacitance bridge testing. These studies indicate the protein that we have characterized and utilized in out surface-immobilization experiments is a high affinity, low capacity pump. These properties are similar to the pump responsible for the buffering of neuronal, intracellular Ca^{2+} during neurotransmitter release(1).

The question of stoichiometry remains problematic due to the fact that the purified ATPase consists of two proteins which stain bands of

different molecular weight. At present, we cannot tell which of these species is responsible for hydrolysis of ATP. Subsequent work has entailed optimal immobilization of calmodulin as a linking molecule.

Although much of the biochemical characterization has been brought to completion with regard to the ATPase, we are continuing studies to evaluate more rapid means of isolation and purification of this protein complex. We have not been limited in the amount of protein required for experimental purposes. However, this may become a potential problem in the future emphasizing the need to generate copious amounts via recombinant DNA technology.

Associated with the ATPase activity is a very labile, high affinity, dihydropyridine receptor activity which we have had much difficulty preserving. Recent experiments have indicated successful solubilization using an anionic detergent (CHAPS). Under conditions preserving dihydropyridine binding, ATPase activity has likewise been observed to persist such that both biologic functions can be monitored on surfaces. However, the dihydropyridine ligand binding function will demand continued investigation with regard to basic properties.

Our future emphasis should center about careful defining of the immobilized biologic sensing moiety. We must concentrate on immobilized protein (calmodulin, calmodulin + receptor protein) and determine its stability on the surface under a variety of different conditions. We need to establish conclusively the length of time that the complex remains immobilized. This will most likely be a function of steric phenomenon. Other important parameters include how to present the complex to the surface. Should the receptor protein be configured in defined, vectorially pumping vesicles capable of undirectional pumping of Ca^{2+}? Should we add

linking proteins sequentially and if so, what quantities are optimal? Can we increase the sensitivity by enzymatic amplification?

At this point we can 1) immobilize purified (Ca^{2+} + Mg^{2+})-dependent ATPase and 2) detect changes occurring on the surface either as increases in phosphate released and/or capacitance differences following interaction of the complex with a test ligand. We must now carefully define the immobilized receptor protein such that conditions giving rise to false negatives as well as those giving rise to false positives following interaction with test ligands can be identified.

Table I

Kinetic Summary (μM)

	Low	High
ATP	24.00	3.20
Ca^{+2}	0.24	0.24
Mg^{+2}	110.00	18.00

Table II

Effects of Various Mitochondrial Inhibitors on Low and High Affinity ATPase Activities

	% Inhibition	
	Low	High
Rotenone (12 μM)	15	20
KCN (0.1 mM)	0	0
NaN_3 (0.1 mM)	0	0
Oligomycin (50 μM)	15	10
Ruthenium red (10 μM)	0	0

Table III

Effects of Various Cations on Low and High Affinity ATPase Activities

	Low Affinity % Control	High Affinity % Control
Cs^{+1}	78	0
Na^{+1}	9	91
NH_4^{+1}	9	94
Li^{+1}	57	11
Mn^{+2}	52	0
Cu^{+2}	56	66
Zn^{+2}	71	58
Co^{+2}	34	34
Ba^{+2}	72	132
Fe^{+3}	49	28
Al^{+3}	51	0

Table IV

The Effects of Purified Mojave Toxin on Dihydropyridine Binding

	fmoles bound/mg
A. Synaptic Membranes + ^{3}H-Ligand	198.0
B. Synaptic Membranes + ^{3}H-Ligand + Unlabelled Ligand	7.6
C. Toxin + Synaptic Membranes + ^{3}H-Ligand	7.5
D. Toxin + Synaptic Membranes + ^{3}H-Ligand + Unlabelled Ligand	7.6
E. Toxin + ^{3}H-Ligand	3.8
F. Toxin + ^{3}H-Ligand + Unlabelled Ligand	3.0

Table V

The Effects of 4-Bromophanacylbromide Treatment on (Ca^{+2} + Mg^{+2})-ATPase and Nitrendipine Binding

4-Bromophenacyl-bromide (μM)	% Control ATPase	% Control Nitrendipine Binding
1.0	78	100.0
10.0	54	80.5
100.0	25	30.0

Table VI

Atomic Absorption Data

	Ca^{+2}(mg/L)	Mg^{+2}(mg/L)
Predialyzed	7.3	12.0
Dialyzed	7.0 (175 μM)	7.1 (292 μM)
Detection Limit	2.0	2.0

Numbers in parentheses represent equivalent Ca^{+2} and Mg^{+2} concentrations (μM) following dialysis.

Table VII

Summary of Derived Kinetic Constants

	K_m(μM)
ATP	157.0
Ca^{+2}	0.024
Mg^{+2}	141.0

Table VIII

Nucleosidetriphosphate Specificity

Substrate	% Control Activity
ITP	23.0
CTP	2.0
GTP	15.0
TTP	0
UTP	0

Table IX

Effects of Various Phospholipids on Purified (Ca^{+2} + Mg^{+2})-Dependent ATPase from Bovine Brain

Phospholipids Tested	% Inhibition	% Activation
Phosphatidylethanolamine	0	9
Lysophosphatidylethanolamine, palmitoyl	0	4
Phosphatidyl-L-Serine	0	28
Lysophosphatidyl-L-Serine	0	10
Phosphatidylcholine	0	21
Phosphatidylcholine, Lauroyl	13	0
Lysophosphatidylcholine, Myristoyl	12	0
Lysophosphatidylcholine, Oleoyl	2	0
Phosphatidylinositol	0	19
Lysophosphatidylinositol	0	2
Phosphatidyl-DL-Glycerol	0	18
Phosphatidic Acid	38	0
Lysophosphatidic Acid, Oleoyl	0	0

Table X

Lectin Affinity Chromatography of Purified (Ca^{+2} + Mg^{+2})-Dependent ATPase from Bovine Brain

(% Starting Activity)

Lectin	Wash	Eluate	Total
WGA	60	45	105
UEA-1	51	40	91
SBA	45	72	117
LPA	32	50	82
Con-A	68	38	106

Abbreviation	Sugar Specificity
WGA= Wheat Germ Agglutinin	N-Acetylglucosamine
UEA= Ulex Europaeus Agglutinin	L-Fucose
SBA= Soy Bean Agglutinin	D-Galactose
LPA= Limulus Polyphemus Agglutinin	N-Acetylgalactosamine
Con-A= Concanavalin-A	D-Glucose, D-Mannose

Table XI

All Plates Contained-

Immobilized Ca^{+2} + Mg^{+2}-ATPase, Ca^{++} Channel Complex

Assay conditions:

1. No additions.
2. Buffer + 50 µl Ca^{++} (4µM)
3. Buffer + 50 µl Ca^{++} (4µM) + Calmodulin (300 µg)
4. Buffer + 50 µl Ca^{++} (4µM) + 180 µg Mojave toxin

Capacitance Readings (picoFarads)

Sensor	Baseline	Challenge
1.	28,382	------
2.	10,538	10,168
3.	5,821	5,692
4.	6,763	7,642

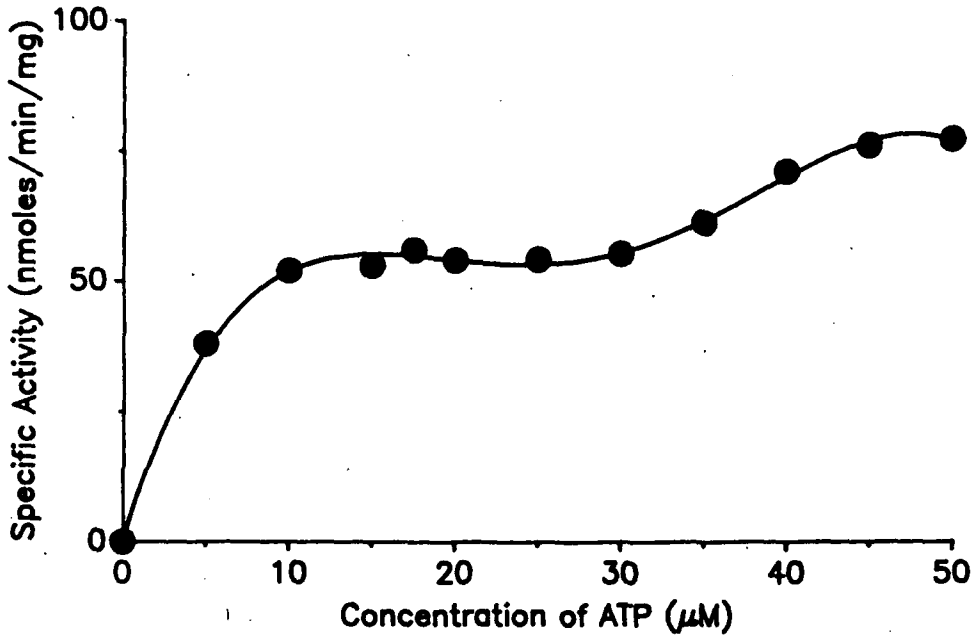


Figure 1. Dependence of ATP Hydrolysis on ATP Concentration in Bovine Brain Synaptic Membranes.

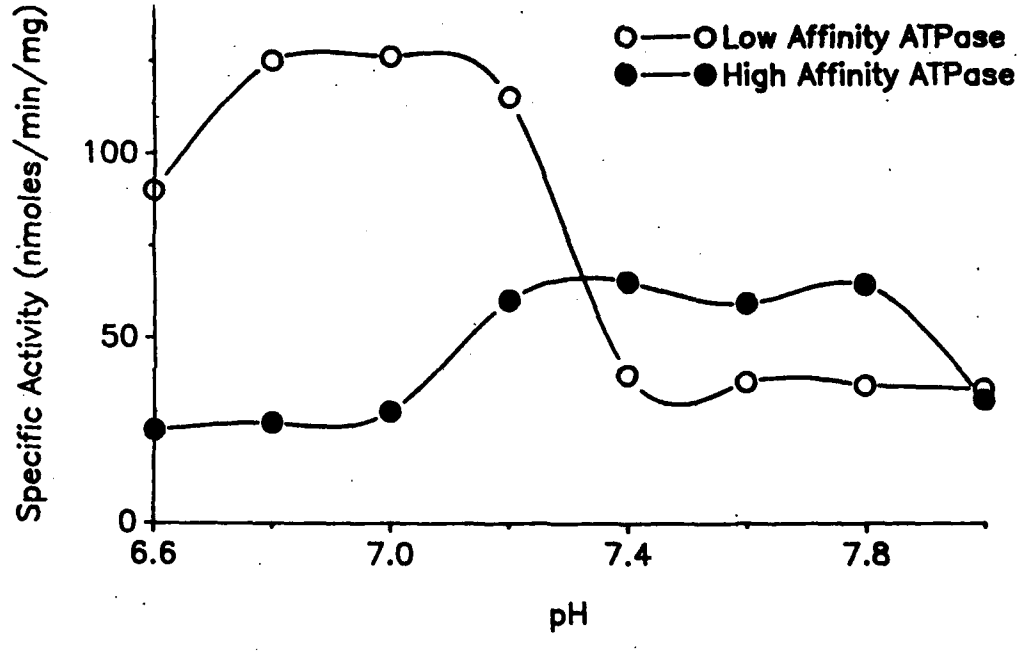


Figure 2. Dependence of ATP Hydrolysis on Hydrogen Ion Concentration in Bovine Synaptic Membranes.

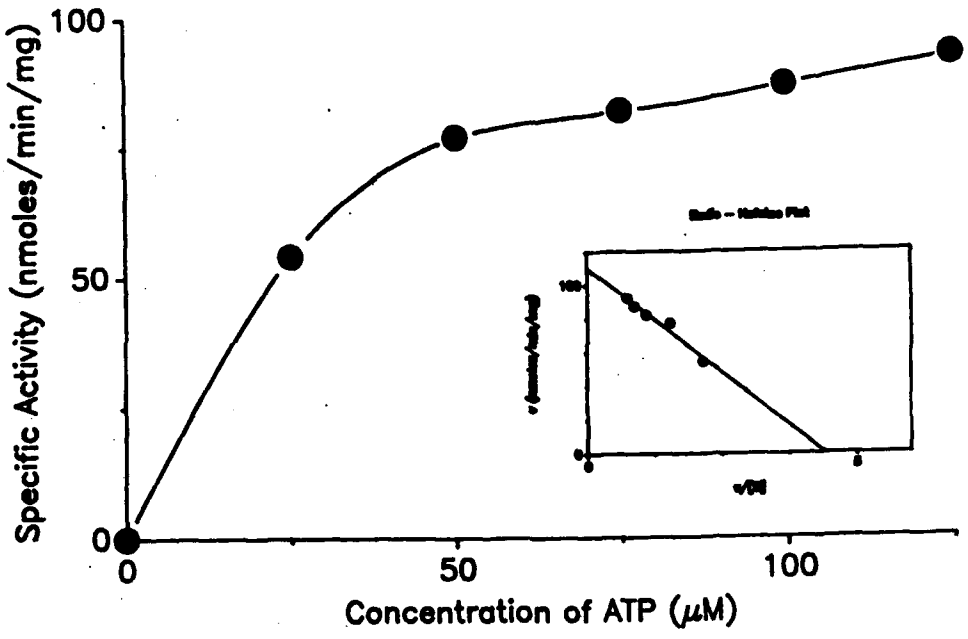


Figure 3. Dependence of ATP Hydrolysis on High Concentrations of ATP.

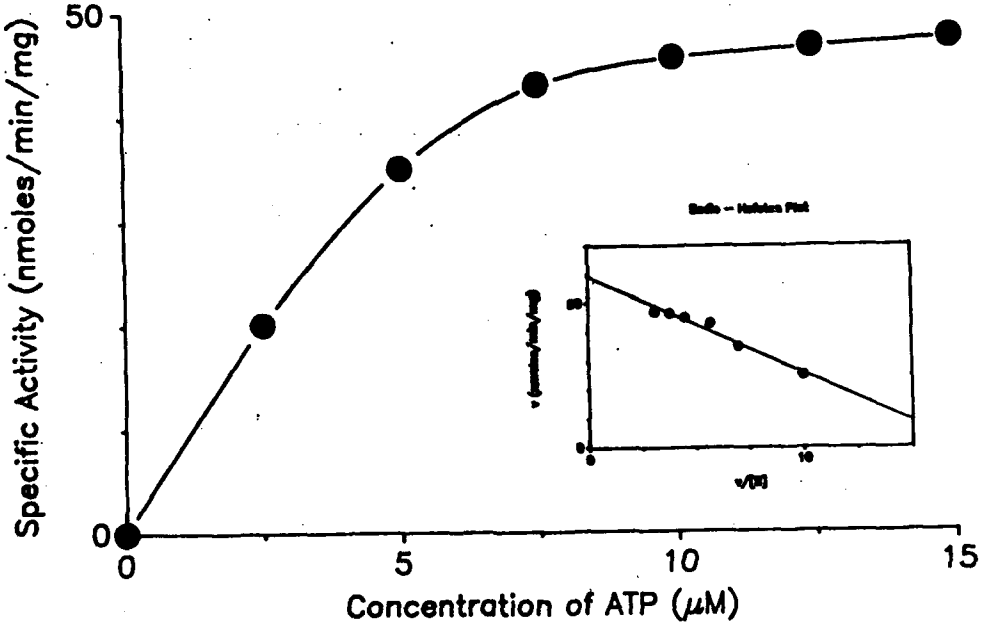


Figure 4. Dependence of ATP Hydrolysis on Low Concentrations of ATP.

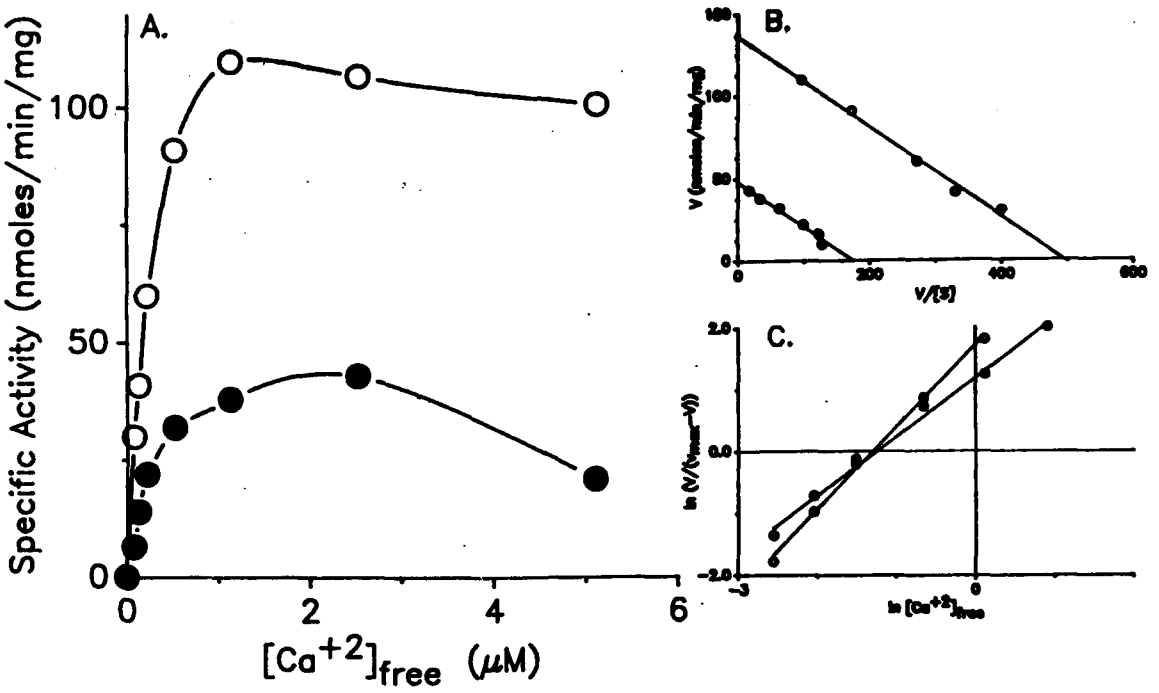


Figure 5. Dependence of ATP Hydrolysis on Ca^{+2}_{free} Concentration.

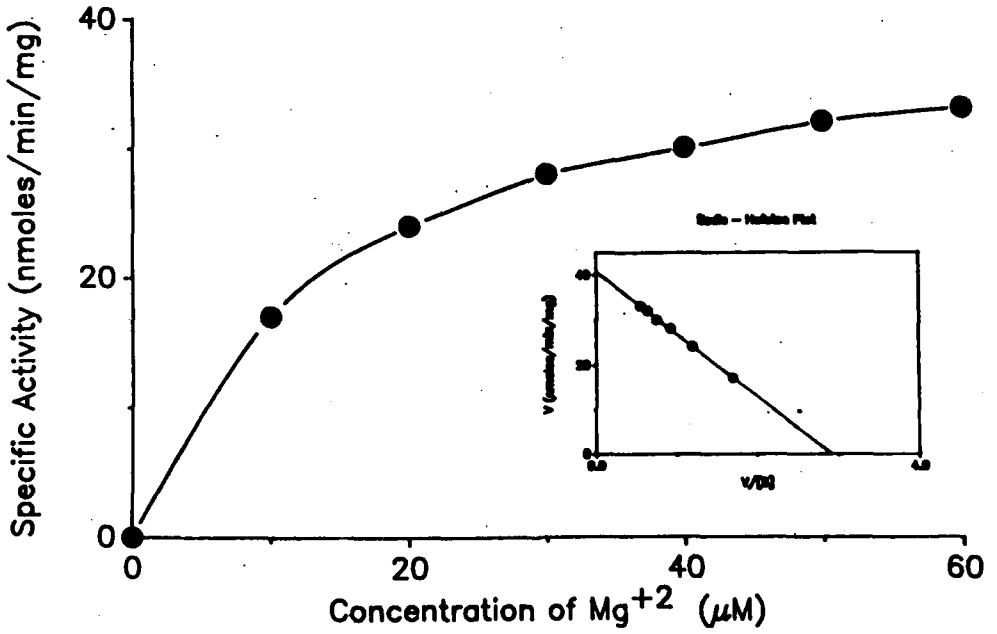


Figure 6. Dependence of ATP Hydrolysis on Mg^{+2} Concentration for the High Affinity ATPase

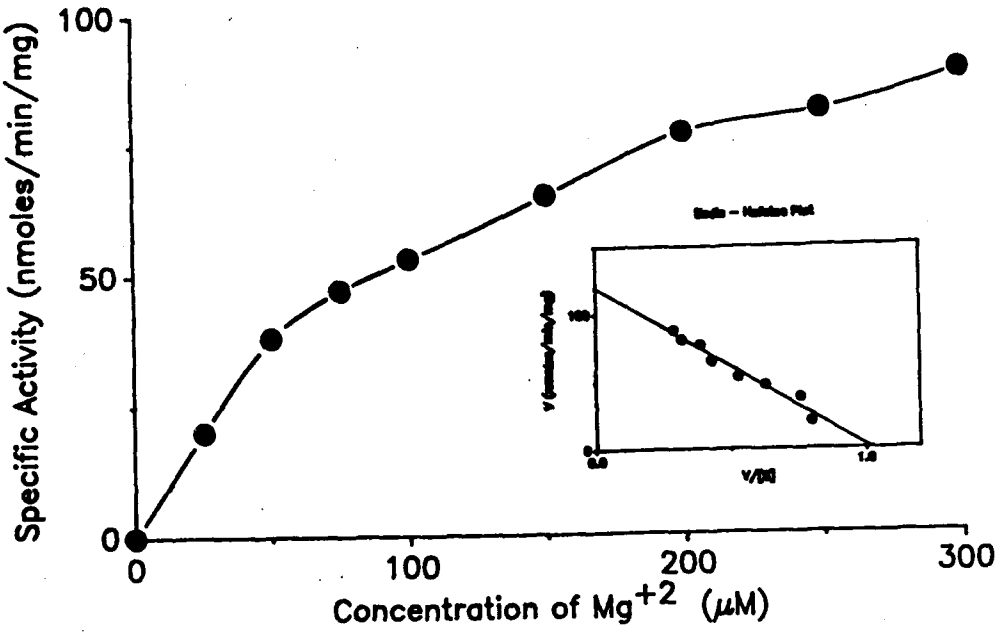


Figure 7. Dependence of ATP Hydrolysis on Mg^{+2} Concentration for the Low Affinity ATPase.

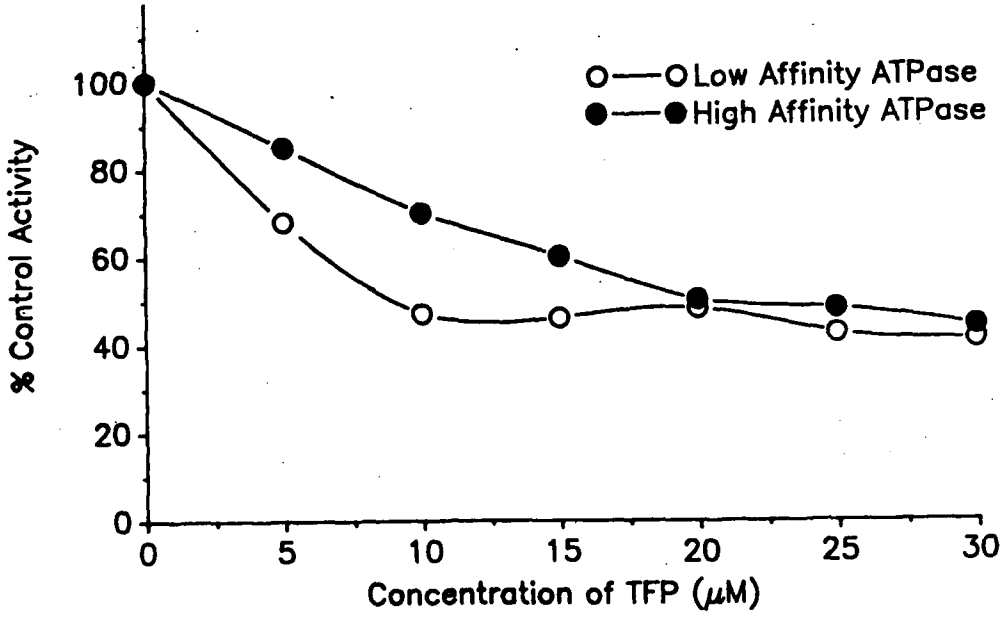


Figure 8. The Effects of Trifluoperazine on $(Ca^{+2} + Mg^{+2})$-ATPase.

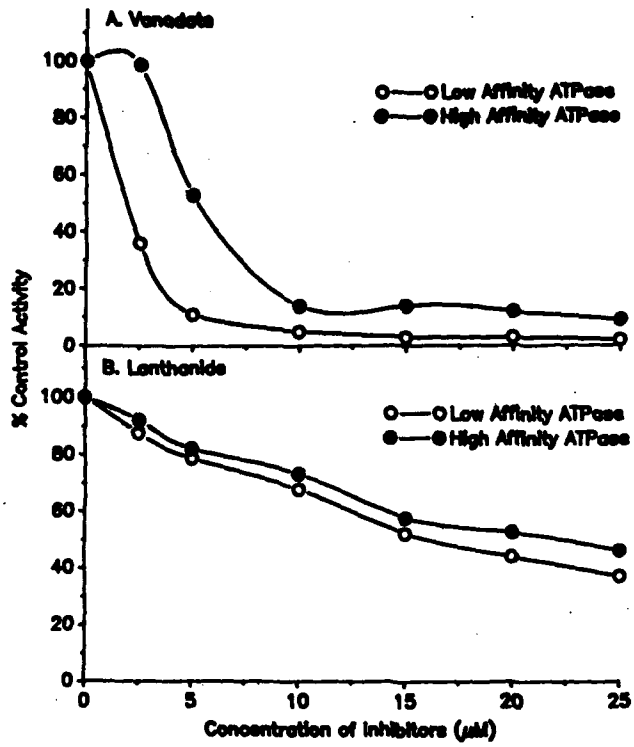


Figure 9. The Effects of the Inhibitors Lanthanide and Vanadate on $(Ca^{+2} + Mg^{+2})$-ATPase.

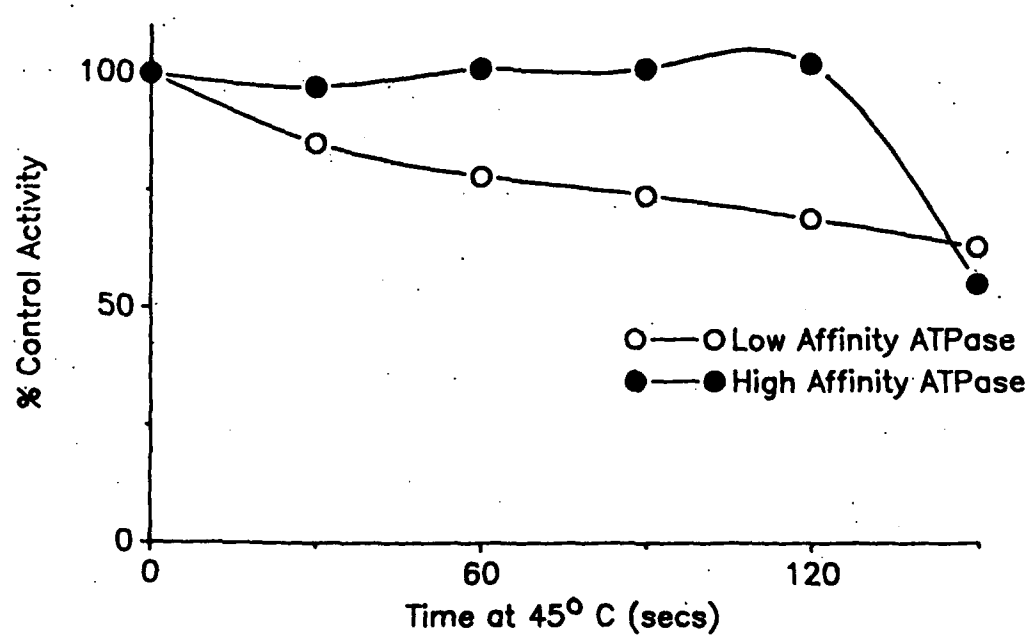


Figure 10. Thermal Lability of $(Ca^{+2} + Mg^{+2})$-ATPase.

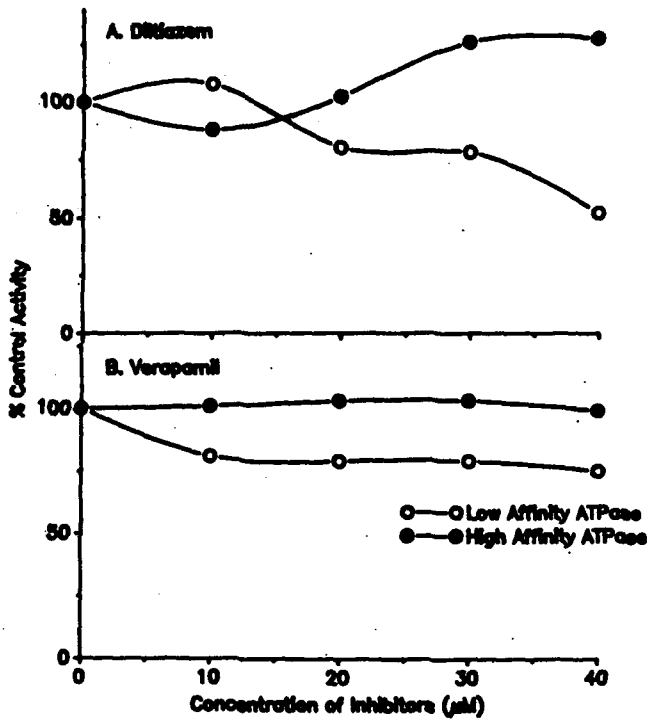


Figure 11. The Effects of Dihydropyridine Binding Modulators on $(Ca^{+2} + Mg^{+2})$-ATPase.

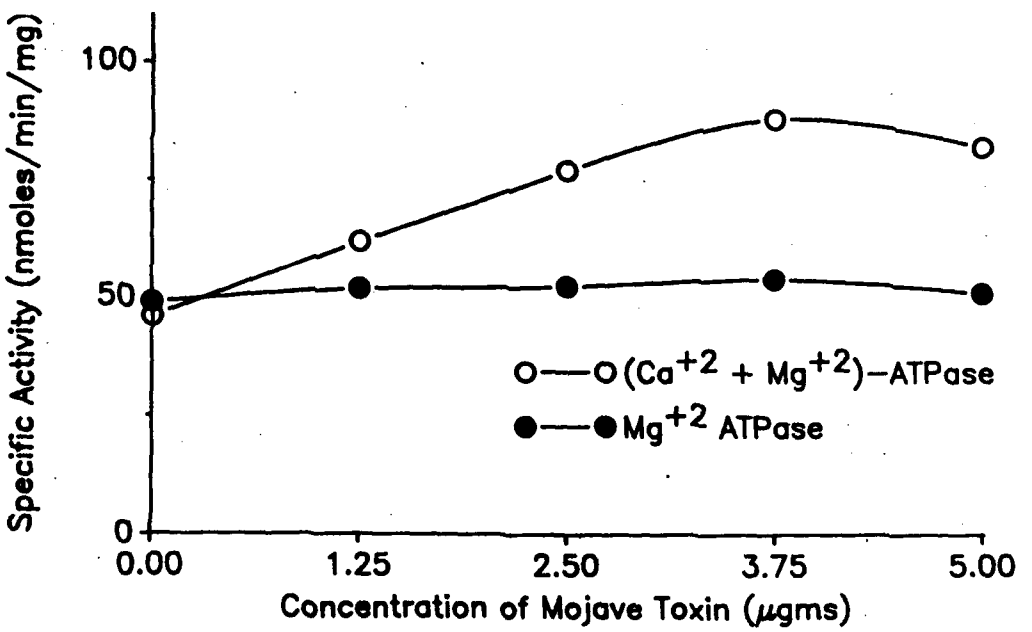


Figure 12. The Effects of Mojave Toxin on ATP Hydrolysis.

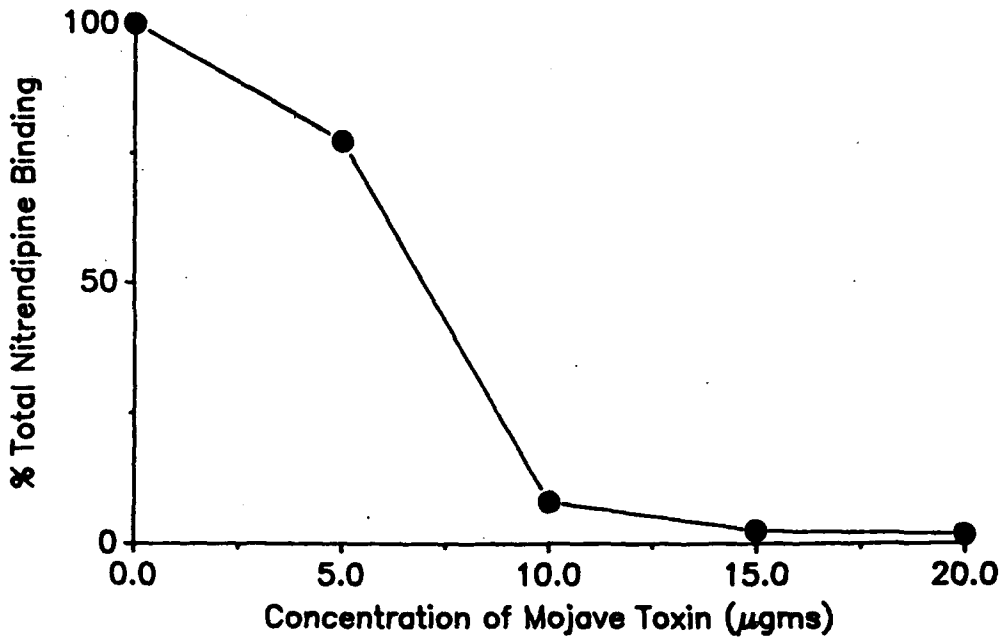


Figure 13. The Effects of Mojave Toxin on Dihydropyridine Binding.

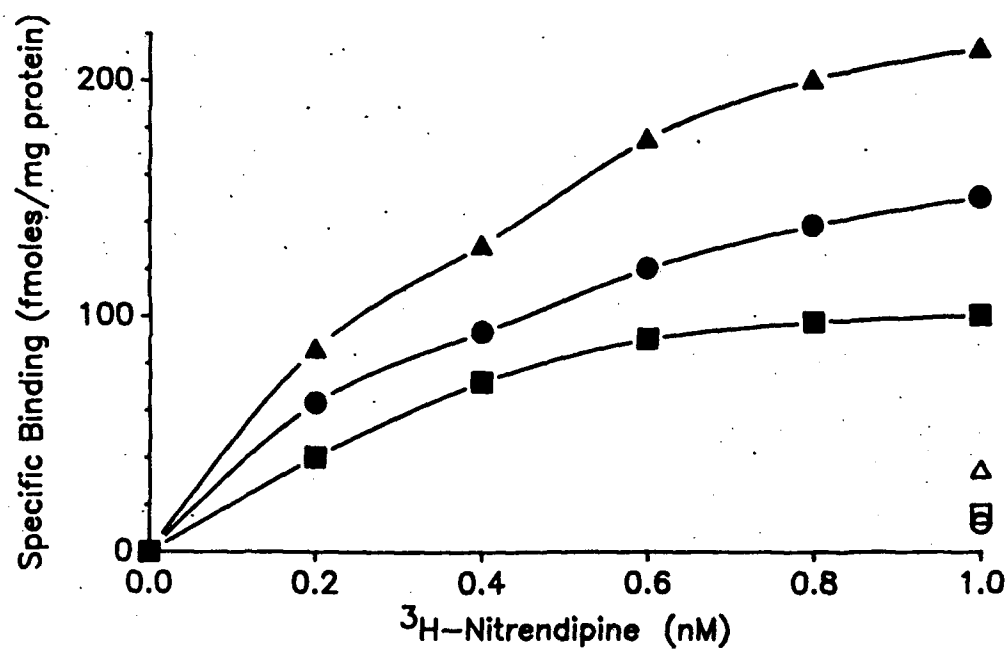


Figure 14. The Effects of Mojave Toxin on the Allosteric Modulators Diltiazem and Verapamil.

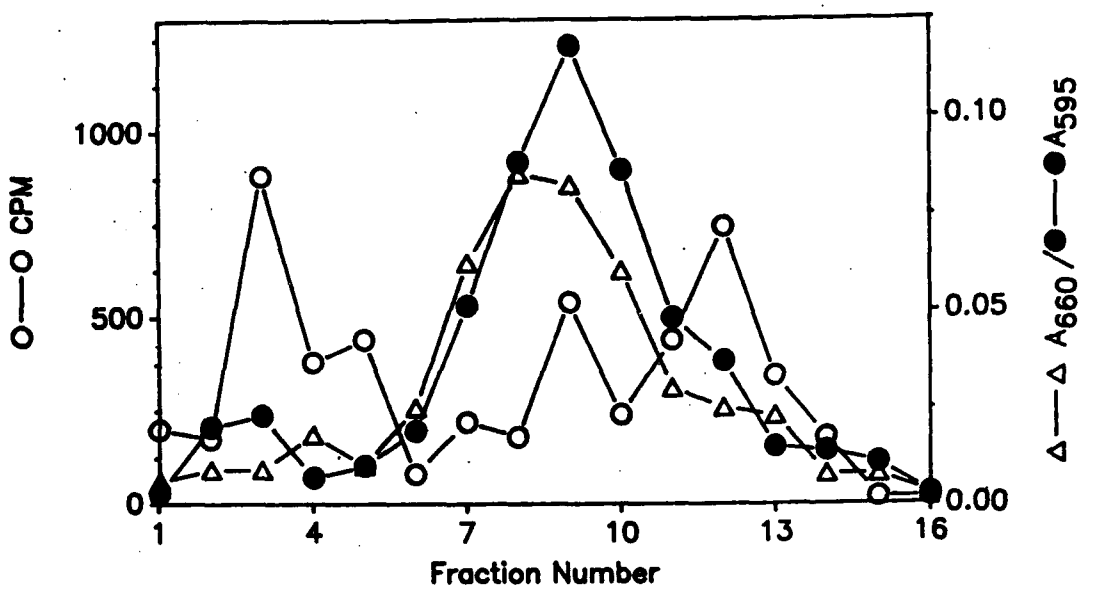


Figure 15. Calmodulin Affinity Column Chromatography of the $(Ca^{+2} + Mg^{+2})$-ATPase and Dihydropyridine Binding Site.

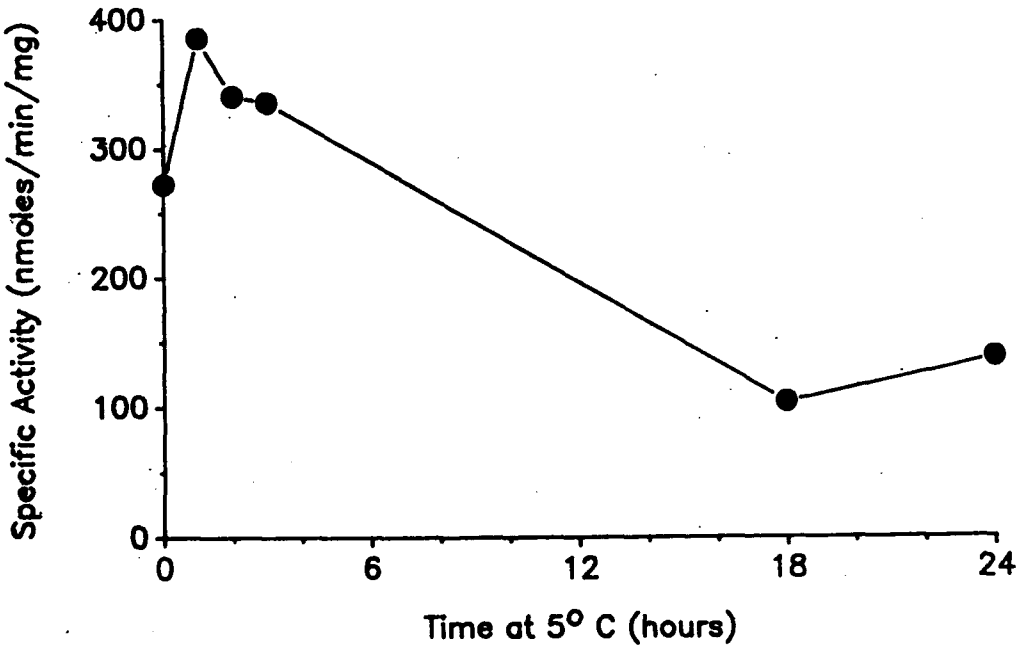


Figure 16. Retention of ATPase Activity Following Dialysis.

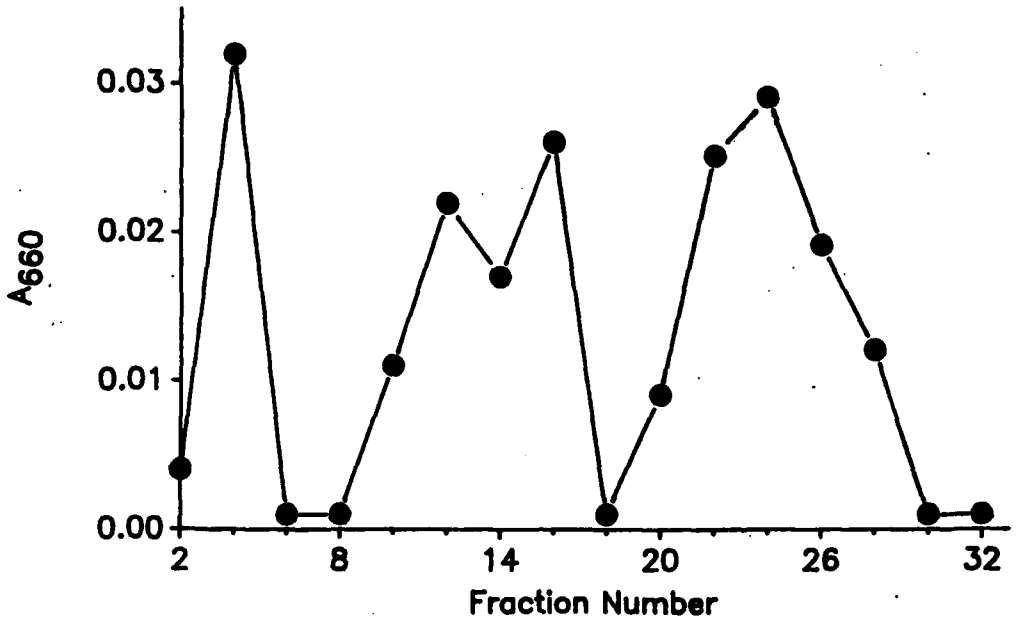


Figure 17. Ion Exchange High Performance Liquid Chromatography.

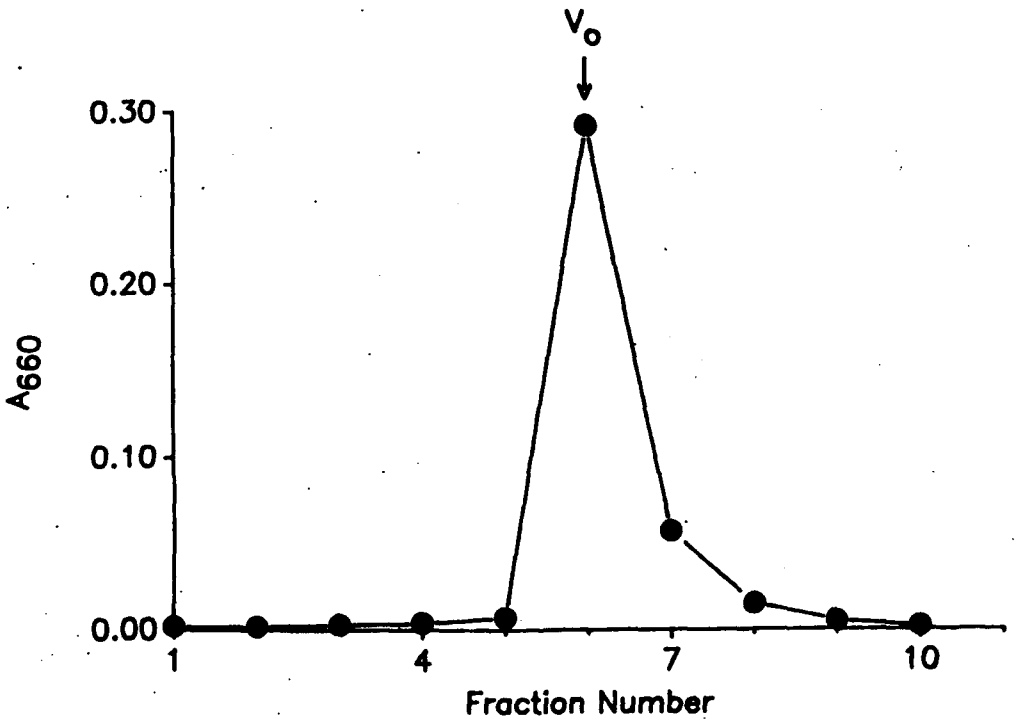


Figure 18. High Performance Liquid Chromatography with a Size Exclusion Column.

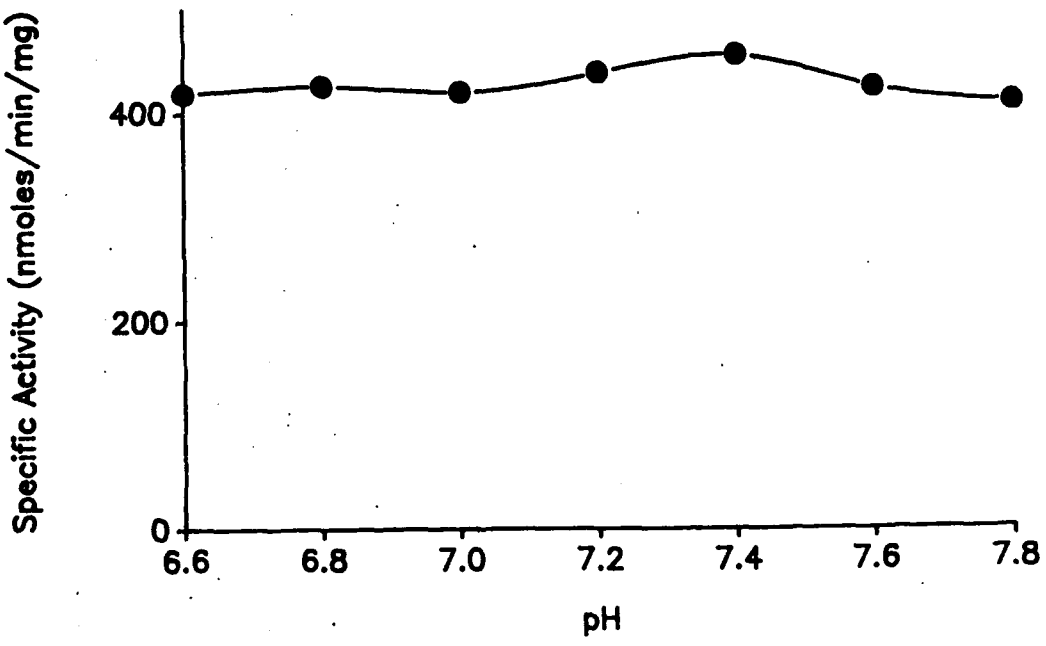


Figure 19. Dependence of ATP Hydrolysis on Hydrogen Ion Concentration.

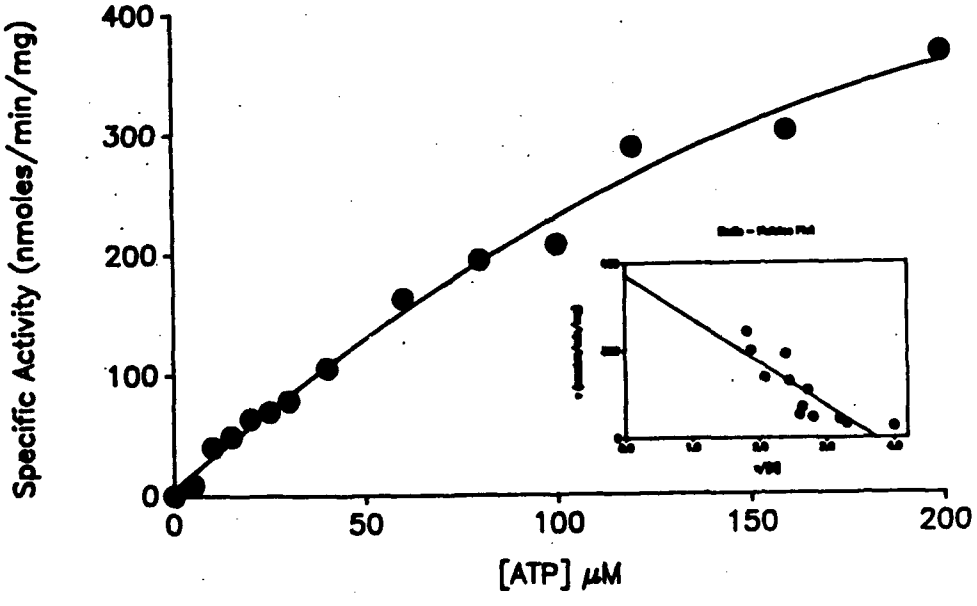


Figure 20. Dependence of ATP Hydrolysis on ATP Concentration.

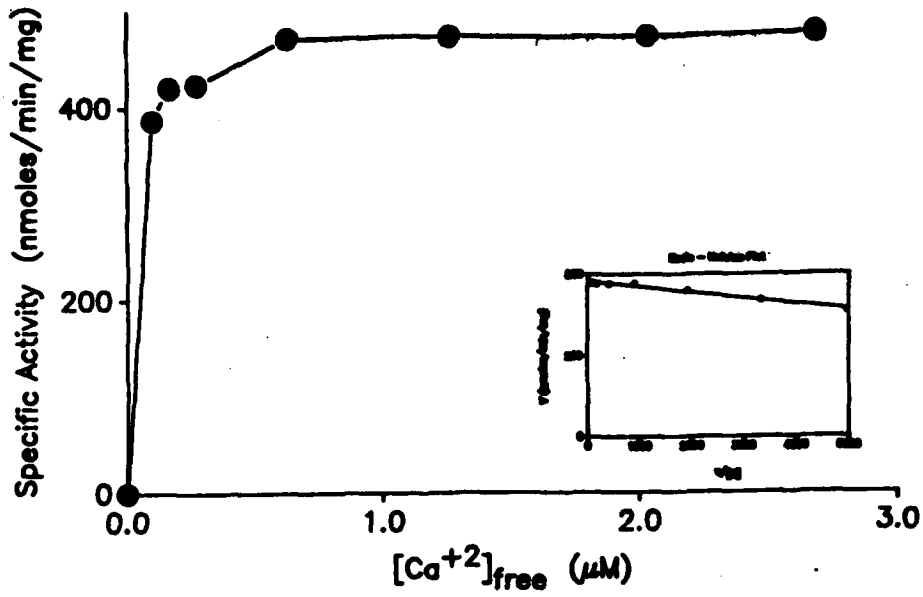


Figure 21. Dependence of ATP Hydrolysis on $(Ca^{+2})_{free}$ Concentration.

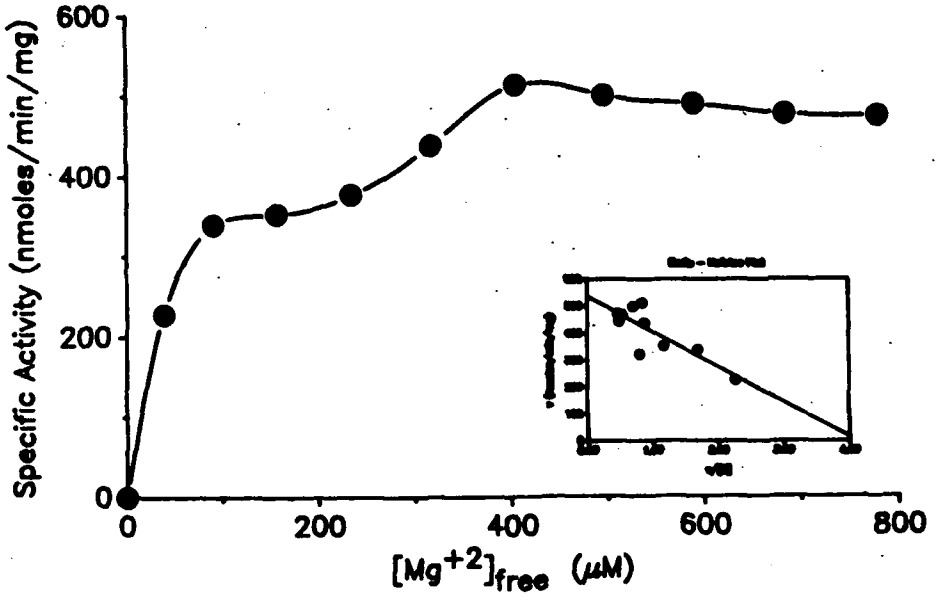


Figure 22. Dependence of ATP Hydrolysis on $(Mg^{+2})_{free}$ Concentration.

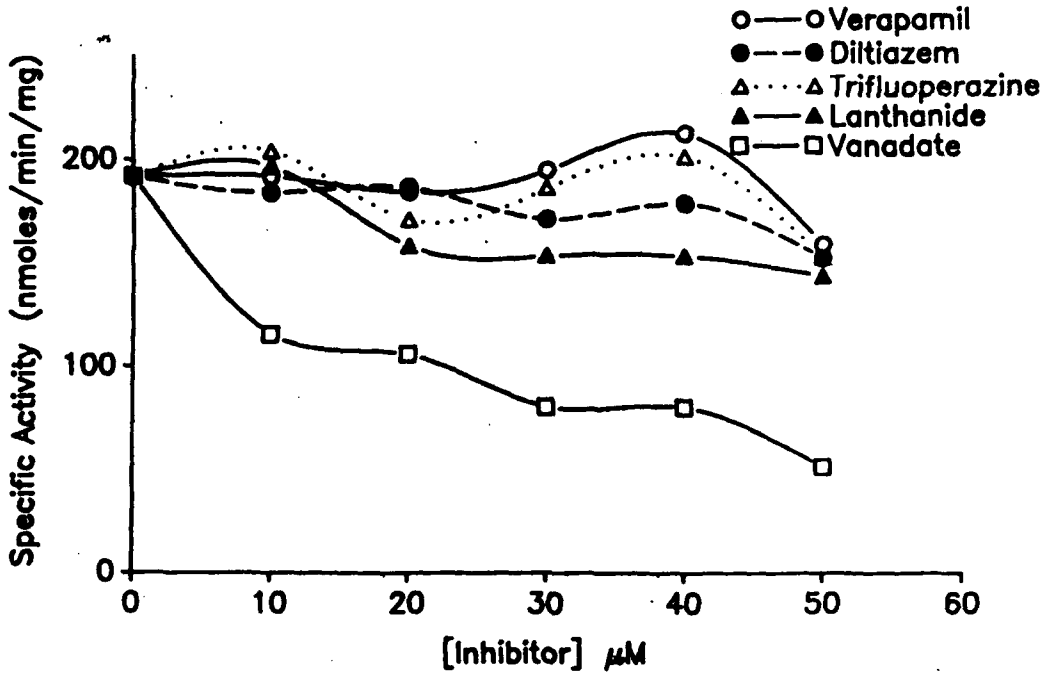


Figure 23. The Effects of Various Inhibitors on (Ca^{+2} + Mg^{+2})-ATPase.

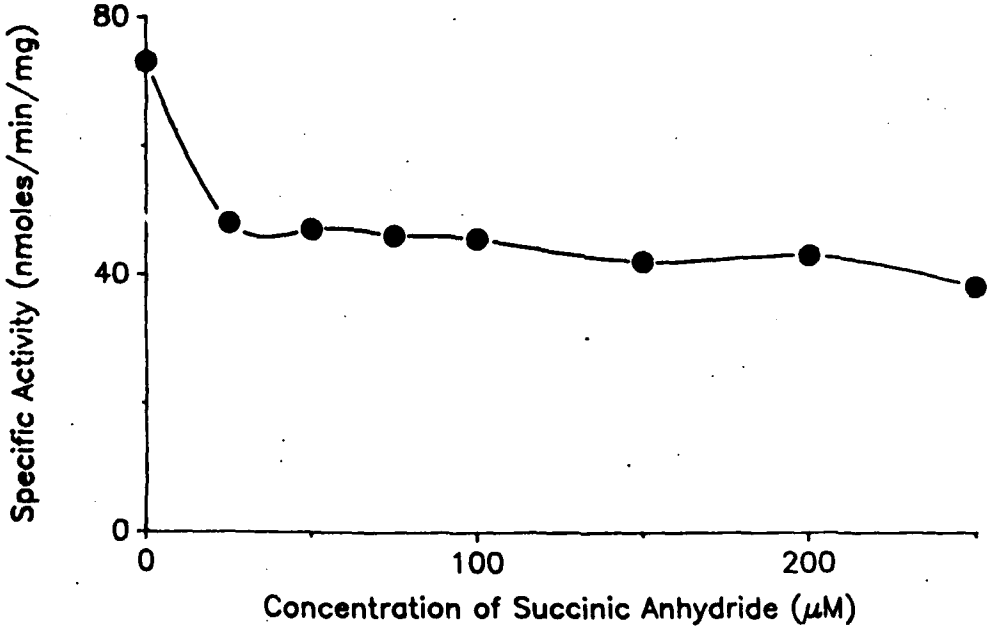


Figure 24. The Effects of Succinic Anhydride on (Ca^{+2} + Mg^{+2})-ATPase.

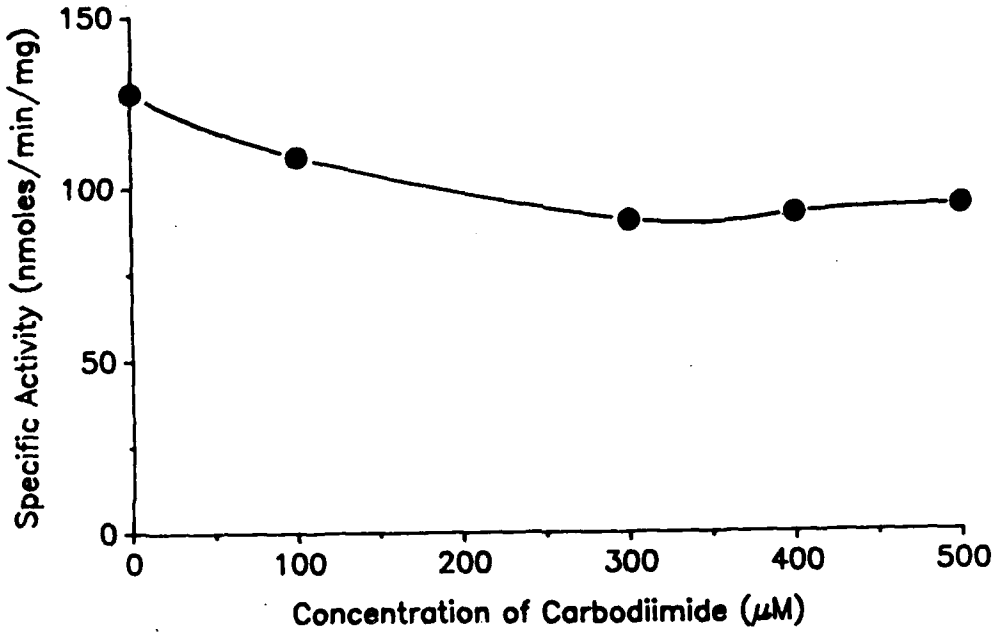


Figure 25. The Effects of Carbodiimide on $(Ca^{+2} + Mg^{+2})$-ATPase.

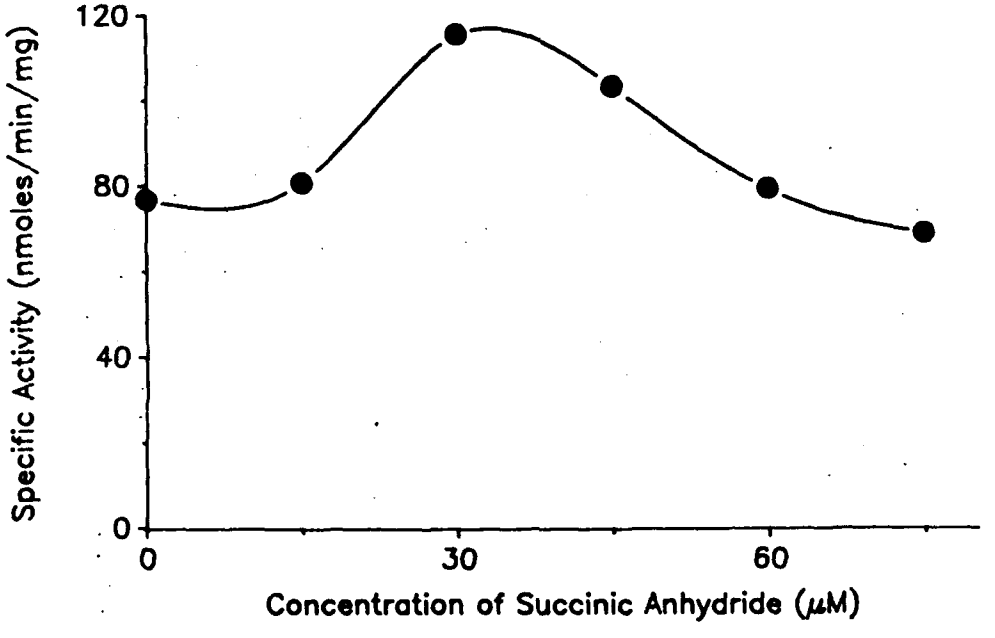


Figure 26. The Effects of Succinic Anhydride on $(Ca^{+2} + Mg^{+2})$-ATPase in Solubilized Enzyme Preparations.

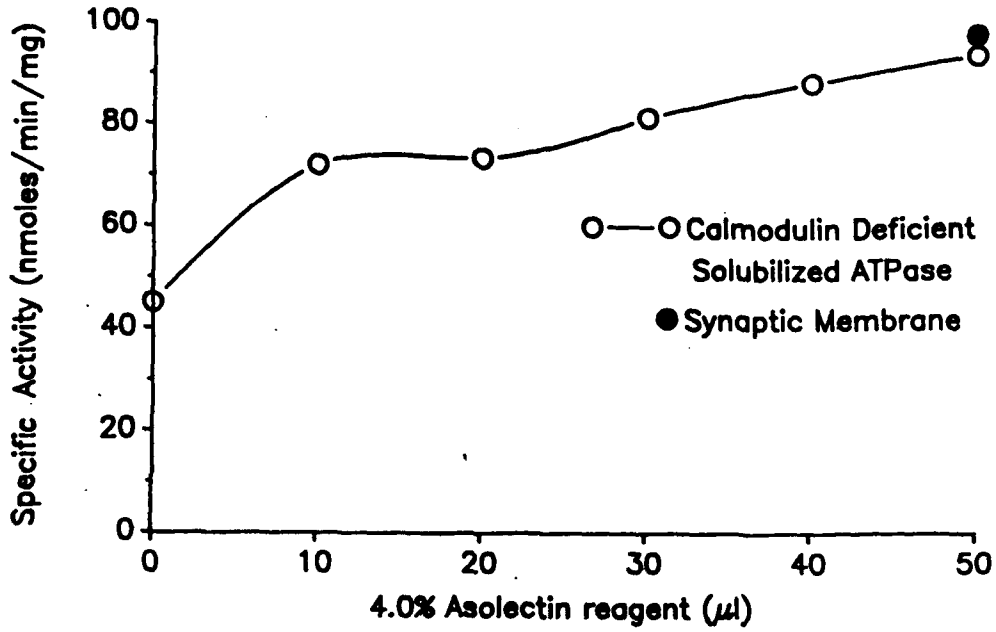


Figure 27. Dependence of ATP Hydrolysis on the Concentration of Asolectin Reagent.

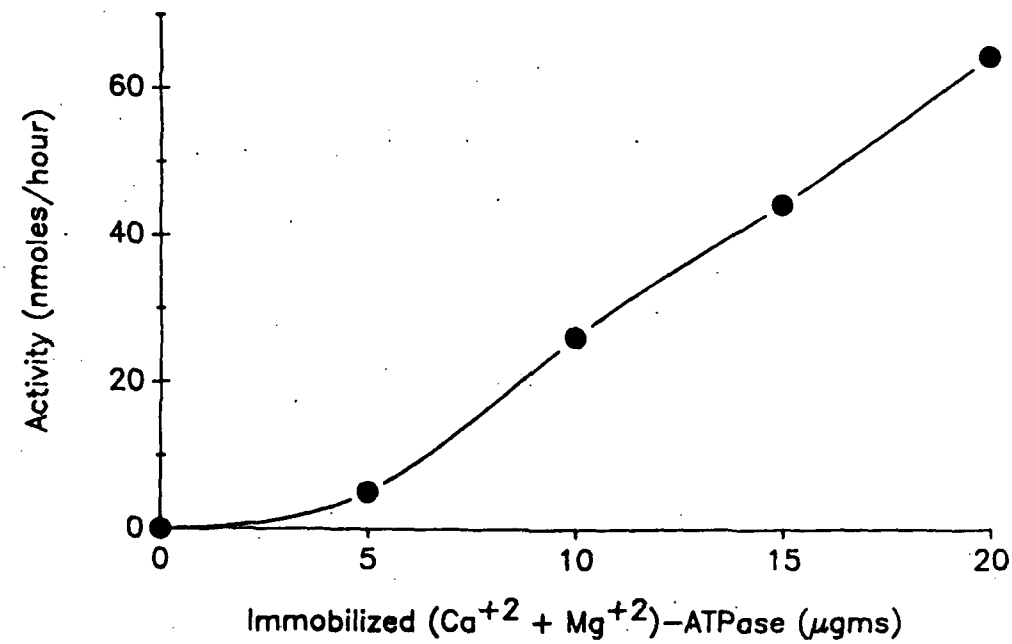


Figure 28. ATP Hydrolysis as a Function of Immobilized $(Ca^{+2} + Mg^{+2})$-ATPase.

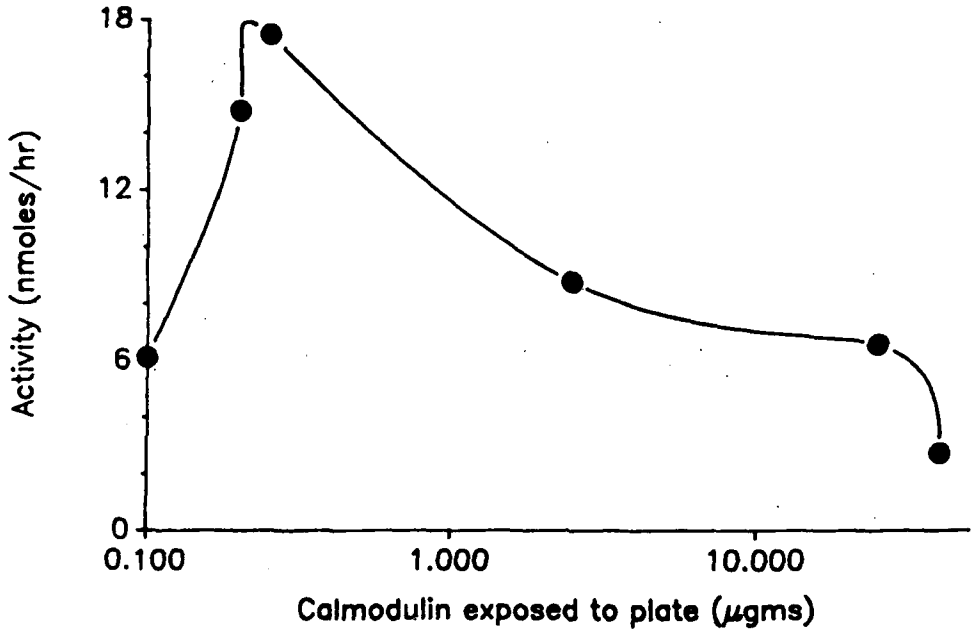


Figure 29. $(Ca^{+2} + Mg^{+2})$-ATPase Adhering to Previously Immobilized Calmodulin.

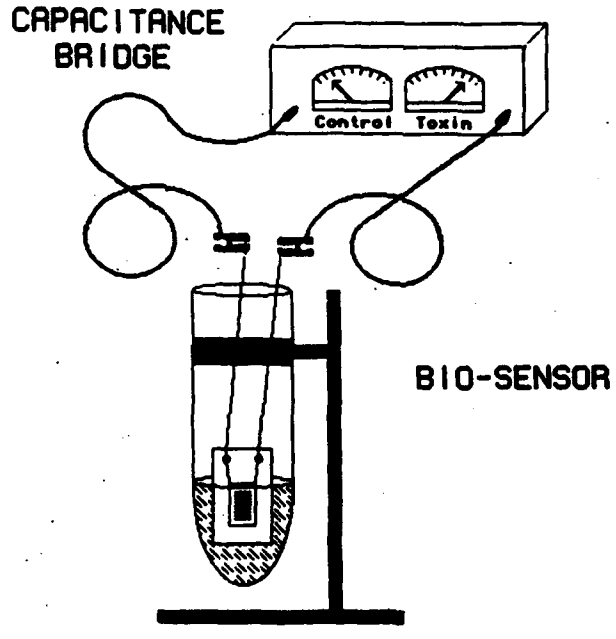


Figure 30. Capacitance Bridge-Based Biosensor.

BLANK

Appendix A

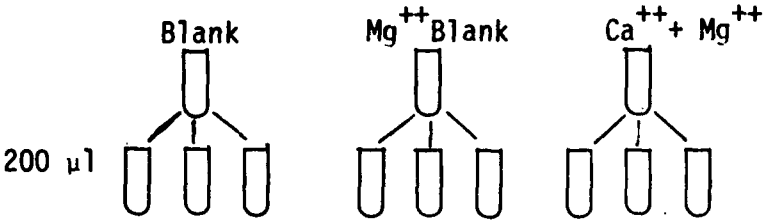
ATP HYDROLYSIS ASSAY

Reagents	Blank	Mg^{++} Blank	Ca^{++} + Mg^{++}
Buffer (0.04 M HEPES, 0.2 M KCl, 0.2 mM EGTA)[2]	1000 µl →		
Ouabain (2 mM)	100 µl →		
Dispersed Phospholipids[1] (Solubilized enzyme only)	50 µl →		
$MgCl_2$[2]		100 µl →	
$CaCl_2$[2]			100 µl
Inhibitors/activators	as required →		
H_2O distilled	Q.S. to 1.8 ml →		
	Incubate at 37°C for 3 minutes		
Protein (1.0 mg/ml)[3]	100 µl →		
	Incubate at 37°C for 3 minutes		
ATP[2]	100 µl →		
	Incubate at 37°C for 1.5 minutes[3]		
HCl (6 N)	200 µl →		
	Assay for phosphate release		

1. 0.4% asolectin, 8.0 mM DTT, 0.2% triton x-100
2. Concentration/pH varied as needed
3. If protein concentration is <50 µg/assay, increase time (e.g. at 5 µg time= 15 min)

PHOSPHATE ASSAY

ATPase assay tubes



Color reagent* 800 µl →

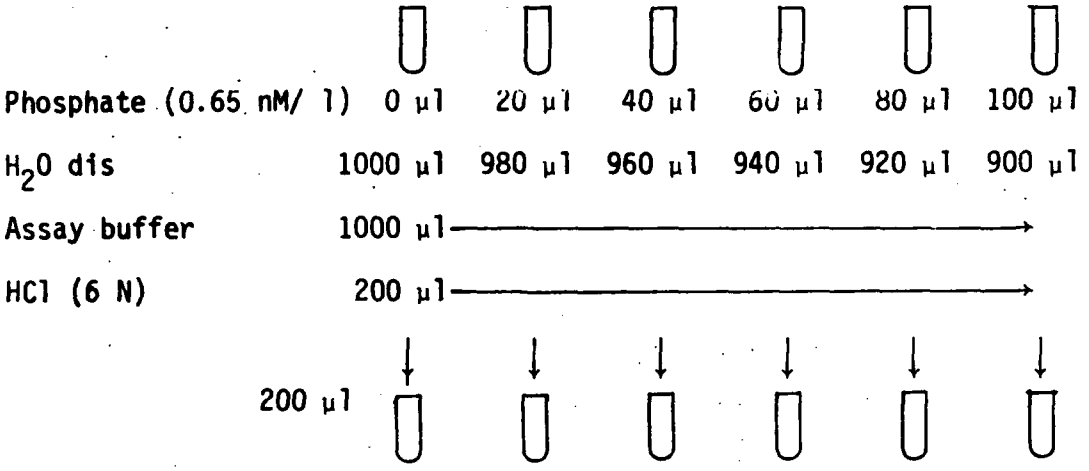
Incubate 60 sec at room temperature

34% Sodium citrate 100 µl →

Incubate 30 min at room temperature
Read at 660nM against a water blank

*Note: 0.045% Malachite green 75 ml
4.2% Ammonium molybdate in 4 N HCl 25 ml
mix 20 min and filter
add 2 ml sterox/ 100ml

Phosphate Standard Curve



Assay for phosphate as above

ISOLATION OF SYNAPTIC PLASMA MEMBRANES

1. 2 g segments of bovine cerebral cortex, dissected so as to be enriched in grey matter, are homogenized in 15 ml of 0.32 M sucrose (all sucrose solutions are prepared in 0.01 M Tris buffer, pH 7.0) by a Potter-Elvejehm homogenizer at 400 rpm for 10 strokes.
2. Centrifuge at 1000 xg for 10 min.
3. Supernatant is carefully decanted into a new centrifuge tube (to include a small amount of the white fluffy layer).
4. Centrifuge at 22,000 xg for 20 min.
5. Pellet is resuspended with hand homogenizer in 10 ml of 0.32 M sucrose. 5 ml of this solution is overlayed on a step gradient made by underlayerin 12.5 ml of 0.8 M sucrose with 12.5 ml of 1.2 M sucrose in a SW centrifuge tube.
6. Centrifuge at 63,000 xg for 60 min.
7. Pipet off the synaptosomal layer (between the 0.8 M sucrose and the 1.2 M sucrose) in 10 ml volume and dilute in 25 ml 0.02 M Tris buffer, pH 8.5, containing 0.5 mM dithioerythritol and 0.1 mM PMSF.
8. Centrifuge at 40,000 xg for 20 min.
9. Resuspend the pellet in 25 ml of the same Tris buffer with homogenization and rotate on ice for 40 min.
10. Centrifuge at 40,000 xg for 20 min.
11. Resuspend the pellets in a total of about 5 ml of 0.01 M Tris, pH 8.2, with 16% glycerol (v/v), and assay for protein concentration.

ISOLATION OF CALMODULIN DEFICIENT MEMBRANES

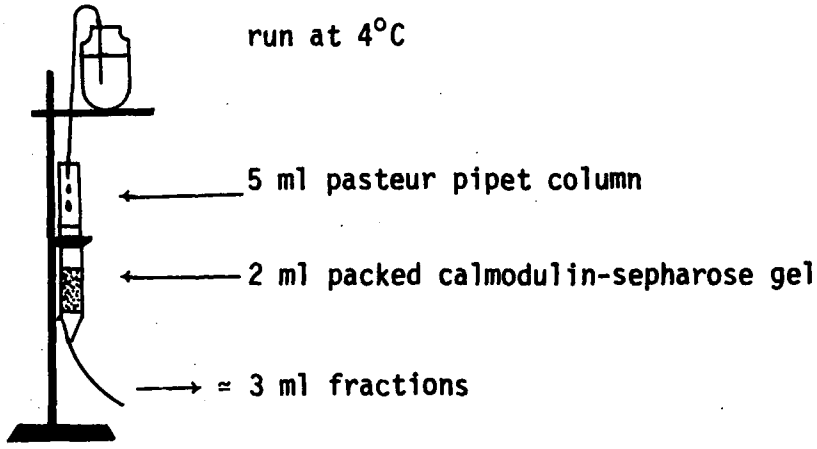
1. Follow steps 1-8 from the isolation of synaptic plasma membranes.
9. Resuspend the pellets in 25 ml of Tris buffer, this time including 2.5 mM EGTA, with homogenization.
10. Centrifuge at 40,000 xg for 20 min.
11. Resuspend the pellets in 25 ml Tris buffer, with 1 mM EGTA, with homogenization and rotate on ice for 40 min.
12. Centrifuge at 40,000 xg for 20 min.
13. Resuspend the pellets in about 4.5 ml of 0.01 M Tris, pH 8.2, with 16% glycerol (v/v).
14. Centrifuge at 100,000 xg for 20 min.
15. Resuspend the pellets in Tris-glycerol and assay for protein concentration.

SOLUBILIZATION OF MEMBRANES

1. To either synaptic plasma membranes or calmodulin deficient membranes in Tris-glycerol is added Triton x-100 at 0.3 mg triton x-100/ mg protein, and solutions are mixed on ice for 45 minutes.
2. Centrifuge at 100,000 xg for 20 min.
3. Supernate is carefully removed and assayed for protein concentration.

AFFINITY COLUMN CHROMATOGRAPHY

1. Calmodulin-agarose gel is poured into a pasteur pipet and equilibrated in at least 10 volumes of buffer (0.01 M HEPES buffer, pH 7.4, containing 0.2 M KCl, 0.1 mM $CaCl_2$, 2.0 mM $MgCl_2$, 2.0 mM DTT, 0.1% asolectin, 0.05% triton x-100)



2. No more that 10 mg of sample are diluted 1:2 with equilibrating buffer and are washed on the column in at least 15 volumes of equilibrating buffer (3 ml fractions are collected).

3. The column is then washed with eluting buffer (0.01 M HEPES buffer, pH 7.4, containing 0.3 M KCl, 1.0 mM EDTA, 2.0 mM DTT, 0.1% asolectin, 0.05% triton x-100) with enzyme normally appearing in the second and/or third fraction.

TREATMENT OF SURFACES

1. Cleaning:

   a. Surfaces are scratched lightly with a diamond point to maintain orientation and are removed from the plastic backing.

   b. They are placed into 10X 75 test tubes containing hot trichloroethylene and are heated for 5 min at 75°C.

   c. They are rinsed in two changes of 95% ethanol (warm) and dried.

2. Derivatization:

   a. 3-aminopropyltrhethoxysilane (APTES) is diluted to a 2% solution in 95% ethanol and is allowed to sit for 5 min to allow for hydrolysis.

   b. Plates, scratched surface uppermost, are covered for two min with the APTES solution, then rinsed with a quick dip in 95% ethanol and allowed to cure for 24 hrs in 10 X 75 test tubes.

3. Calmodulin Derivatization of Surfaces:

   a. Calmodulin is diluted with 10 mM Tris, pH 7.4, to give appropriate concentrations in 25 µl. The Tris contains 200 µM $CaCl_2$.

   b. 25 µl of calmodulin solution is placed on the scratched surface of APTES-treated surfaces. Blanks with just Tris are also prepared.

   c. 10 µl of 1 mM cyanimide in 3 M NaCl is then added to each surface.

   d. Surfaces are incubated at 4°C for two hours.

   e. The surfaces are washed by dipping in four changes of 10 mM Tris, pH 7.4, containing 0.02% triton x-100 and 1 M NaCl, followed by dipping one time into the same Tris solution without NaCl.

LITERATURE CITED

1. Blaustein, M.P., Ratzloff, R.W., and Schweitzer, E.S. (1978) J. Gen. Physiol. 72, 43-66.

2. Wieland, G. and Oswald, R. (1985) J. Biol. Chem. 257, 7287-7289.

3. Spamer, C., Heilmann, C. and Gorek, W. (1987) J. Biol. Chem. 262, 7782-7789.

4. Kendrick, N.C., Blaustein, M.P., Fried, R.C. and Ratzloff, R.W. (1977) Nature 265, 246-248.

5. Papazian, D., Rahamimoff, H. and Goldin, S.M. (1981) Soc. Neurosci. Abstr. 7, 367.

6. Lewin, R.M. and Weiss, B. (1978) Biochim. Biophys. Acta. 540, 197-204.

7. Sobue, K., Ichida, S., Yoshida, H., Yamazaki, R. and Kakiuchi, H. (1979) FEBS Lett. 99, 199-202.

8. Sorenson, R.G. and Mahler, H.R. (1981) J. Neurochem. 37, 1407-1418.

9. Michaelis, E.K., Michaelis, M.L., Chang, H.H. and Kitos, T.E. (1983) J. Biol. Chem. 258, 6101-6108.

10. Pershadsingh, H.A. and McDonald, J.M. (1981) J. Biol. Chem. 255, 4087-4093.

11. Lotersatajn, S. and Pecker F. (1982) J.Biol. Chem. 257, 6638-6641.

12. Ehlert, F., Roeske, W., Itoga, E. and Yomamura, H. (1982) Life Sci. 30, 2191-2202.

13. Yamamura, H., Shoemaker, H., Boles, R. and Roeske, W. (1982) Biochem. Biophys. Res. Commun. 108, 640-646.

14. Rael, E.D., Salo, R.J. and Zepeda, H. (1987) Toxicon, in press.

15. Cate, R. and Bieber, A. (1978) Arch. Biochem. Biophys. 189, 397-408.

16. Roberts, M., Deems, R., Mincey, T. and Dennis, E. (1977) J. Biol. Chem. 252, 2405-2411.

17. Valdes, James J., Wolff, Vicki L., Menking, Darrel E., Thompson, Roy G., Rael, E.D. and Chambers, James P. Ion channel binding specificity to Mojave toxin. Submitted to Toxicon, 1988.

18. Niggli, V., Penniston, J.T. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955-9958.

19. Laemmli, U.K. (1970) Nature(London) 227, 680-685.

20. Merril, C.R., Dunan, M.L. and Goldmann, D. (1981) Anal. Biochem. 110, 201-207.

21. Goldin, S.M., Chan, D.M., Papazain, E.J., Hess, E.J. and Rahamimoff, H. (1983) Cold Spring Harbor Symp. Quant. Biol. 48, 287-295.

22. Bartfai, T. In: Advances in Cyclic Nucleotide Research, Vol. 10, ed. Brooker, G., Greengard, P. and Robison, G.A. New York: Raven Press, 1979.

23. Fabiato, A. and Fabiato, E. (1979) J. de Physiologie, Paris 75, 463-505.

24. Ikemota, N., Garcia, A.M., Kwobe, Y. and Scott, T.L. (1981) J. Biol. Chem. 256, 8593-8601.

25. Stahl, N., and Jencks, W.P. (1987) Biochemistry 26, 7654-7667.

26. Debray, H., Decout, D., Strecker, G., Spik, G. and Montreuil, J. (1981) Eur. J. Biochem. 117, 41-55.

27. Yariv, J., Kalz, A.J. and Levitski, A. (1968) Biochem. Biophys. Acta 165, 303-311.

28. Davey, M., Huang, J.W., Sulkowski, E. and Carter, W.A. (1974) J. Biol. Chem. 249, 6354-6355.